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SEX DIFFERENTIATION IN GONADS DEVELOPED FROM TRANSPLANTS OF THE INTERMEDIATE MESODERM OF *AMBLYSTOMA*.

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INTRODUCTORY AND HISTORICAL.

From his studies on parabiotic twins in *Amblystoma punctatum* Burns ('25) is led to the conclusion that in this species there may occur a complete reversal of sex previous to the period of sex-differentiation. Embryos joined in pairs in early stages should, by the laws of chance, be combined in the proportion of 1 ♂♂ : 1 ♂♀ : 1 ♀♂ : 1 ♀♀. Instead of this expected ratio, Burns obtains exclusively one-sexed pairs, in the proportion of 44 ♂♂ to 36 ♀♀. This result he is inclined to interpret as a 1 : 1 ratio. Having no evidence that the two-sexed pairs had been eliminated through selective mortality, Burns postulates that half the pairs reared must have been, originally, ♀♂ combinations; in these pairs, from a condition of near-equilibrium as regards sex, one or the other sex, he assumes, had eventually gained the ascendency, so that at sex-differentiation the gonads of the two members of the pair were identical. Since the sex-ratio found was approximately 1 ♂♂ : 1 ♀♀, Burns infers that there can be no prepotency constantly favoring either male or female, since in this event a 3 : 1 ratio favoring the prepotent sex would be expected.

The more recent studies of Witschi ('27) on frog embryos joined in parabiosis show that in these amphibia the early sex reversal assumed by Burns does not occur. Witschi finds in 56

pairs the following combinations: ♂♂, 16 pairs; ♂♀, 17 pairs, with 7 of the females undergoing sex-reversal; ♀♂, 10 pairs, with 4 of the females undergoing sex-reversal; ♀♀, 13 pairs. This approximates very closely the expected ratio of 1 ♂♂ : 1 ♂♀ : 1 ♀♂ : 1 ♀♀, and shows conclusively that there could have been no sex-reversal previous to the time of sex-differentiation. From the fact that in many of the two-sexed pairs the females were found undergoing sex-reversal, while a female united with a male undergoing reversal was never found, Witschi concludes that the male is always dominant in the sex-reversal which finally occurs. Though he believes that the independent sex-differentiation in the individuals of genetically two-sexed pairs favors the theory of localized sex-differentiators ("lokalisierte Innenfaktoren," probably comparable to Spemann's "Organisatoren"), he states that in the later sex-reversal of the female of the pair, "the coöperation of hormones is not improbable."

The method of parabiosis used by Burns and Witschi has certain obvious disadvantages. If used with a species in which the zygotic sex-determination can be completely reversed previous to sex-differentiation, as is possibly the case in *Amblystoma*, there can be no certainty regarding the original state of any one-sexed pair examined after sex-differentiation has taken place. In drawing conclusions as to the occurrence of sex-reversal in these one-sexed pairs, one must depend entirely upon the sex-ratio obtained. Further, if the death rate among pairs joined in parabiosis is high, the possibility of a selective mortality cannot be entirely eliminated, even though evidence in favor of it may be scanty or lacking. Hence there is no absolute proof that the one-sexed pairs found at sex-differentiation were not all of this character genetically at the time they were joined; the proof of sex-reversal, therefore, remains inconclusive.

The method of parabiosis is relatively advantageous if used with a species in which an early reversal of sex does not occur (as *Rana sylvatica*; Witschi, '27). In such a species, pairs preserved at a suitable period in development would show the actual progress of sex-reversal in one member of the pair. If, however, the reversal becomes complete, all pairs killed at later periods would be found to be one-sexed. Although sex-reversal could be confidently asserted for a species of this type as a result

of the disappearance of the two-sexed condition observable in younger pairs, the identity of any of the originally mixed pairs could be established in adult animals only with great difficulty if at all.

In the spring of 1926 the writer undertook to transplant the intermediate mesoderm of *Amblystoma* from one embryo to a latero-ventral site in another in order to determine the fate of the primordial germ cells included in such grafts. Among the seven embryos surviving the implantation was one in which at forty-four days after operation the germ cells of the graft were found to have given rise to a gonad of considerable size (Humphrey, '27). This suggested the possibility that such grafts, if allowed to develop until after the period of sex-differentiation of the host, might be found to contain gonads which had likewise undergone sex-differentiation. The donor serving as the source of the transplant, and the host into which it was engrafted, though selected at random long before sex-differentiation had occurred, must in many cases be unlike in sex. Since the donor could be reared, its sex could be determined from the gonad it possessed, and since donor and host were not joined, the sex-differentiation in either could not be influenced by the other, except in so far as the graft might be able to modify the sex-differentiation of the host. If, then, after sexual differ entiation the gonad of the graft were found to agree in type with that of the host regardless of the sex of the donor, the fact of an early sex-reversal would be established beyond question. If, on the other, hand, the gonad of any graft differed in type from that of the host, agreeing with that of the donor, it would show conclusively that sex-reversal previous to sex-differentiation had not occurred. By the method of grafting, therefore, it seemed possible to obviate certain difficulties inherent in the method of parabiosis. The donor furnishing the graft would undergo sex-differentiation according to the factors present in the egg at fertilization; its gonad could be compared directly with the gonad developed in a transplant removed during the germ-layer stage and grown in a host of the opposite sex. Conclusions as to the occurrence of sex-reversal, therefore, could be drawn from comparison of structures rather than by reasoning

from sex ratios in which the factor of selective mortality might possibly be involved.

Material and Methods.

The removal of the intermediate mesoderm (preprimordia of gonad and mesonephros) of *Amblystoma* and its implantation into another embryo is a relatively simple operation, the technique for which has been outlined elsewhere (Humphrey, '27). During the operating season of 1927, 180 such implantations were carried out. The graft always included a large part of that region of the intermediate mesoderm in which it had been found that primordial germ cells develop (*i.e.*, the territory of the seventh to the seventeenth somites, approximately); in addition it included parts of the adjacent axial and lateral mesoderm, together with the overlying ectoderm.

Following operation, the host receiving the transplant and the donor furnishing it were reared to the age of fifty days or over—*i.e.*, until after the beginning of morphological sex-differentiation. At autopsy of the host the graft derivatives were found, as a rule, attached to the inside of the ventral or lateral body wall. In the donor, at autopsy, the gonad was always very small or entirely lacking on the right, the side from which the transplant was invariably taken in the embryo.

Results.

Of 180 pairs (donor and host) only 49 or 27 per cent. of the total, were reared to the age of 50 days or over. This, however, does not indicate an actual mortality of 73 per cent. in the grafted animals, since 25 additional hosts were reared to the age of 50 days or more, although the donors which had furnished them transplants had died in early stages of development. Several hosts were also killed before reaching the age of 50 days, in order to study the development of the gonad and other structures in the graft; these hosts were always those of pairs from which the donor had already died from operative injury or other causes. In all, 74 grafts were recovered after sex of the host had become distinguishable. Of these grafts, 40 contained a gonad, the sex of which was determinable with a fair degree of certainty in 33 cases. In the remaining grafts the

gonad was small with few germ cells and no features permitting it to be classified as either ovary or testis.

Unfortunately for this study, the majority of the gonads which developed were in homoplastic transplants in *Amblystoma jeffersonianum*. In this species, instead of the expected 1 : 1 sex-ratio, the animals reared in the laboratory in 1927 were in the proportion of 56 females to 19 males, essentially a ratio of 3 : 1. As a result of the predominance of females, donor and host were both of this sex in an excessive number of cases. In only two instances were donor and host unlike in sex and in these, unfortunately, the gonad of the graft was in each case of somewhat atypical structure due to unfavorable environmental factors.

To the writer's knowledge a sex-ratio such as the one here reported for *Amblystoma jeffersonianum* has not been previously recorded for this species. Whether it is to be explained on the basis of a selective mortality among operated animals, or whether it is due to an induced reversal of sex in certain males resulting from nutritive disturbance or other alteration of environmental factors, or whether an excess of females is a normal condition in this species or at least in its local strain, cannot be positively stated. It is worthy of note that in *Amblystoma maculatum* (*punctatum*) reared in the laboratory under identical conditions and after similar operative procedure, the sex-ratio is apparently quite normal. The collection of large numbers of *A. jeffersonianum* larvæ from local ponds and a study of their sex-ratio has not been possible. The few specimens picked up near ponds after metamorphosis have been found to be females in the great majority of cases.

A second feature of interest noted particularly in this species is the occurrence of spermatocyte stages in the testes of males 60 to 80 days of age. This cannot be due to the presence of a graft furnished by a female, since spermatocytes are no more frequent in hosts than in donors. Though Burns ('25) makes no mention of spermatocytes in *A. maculatum* of similar age, the writer has encountered such stages occasionally in this species as well as in *A. jeffersonianum*. In the latter, however, they occur in a higher percentage of the males examined, and usually in greater numbers than in *A. maculatum*. In neither species, were the spermatocytes found in stages later than the pachytene

condition of the heterotypic prophase. Though the presence of heterotypic prophases in males renders these stages of little value as a criterion of sex when considered alone, it may be noted that their number in the male is small as compared with the number of other germ cells, and that they were not found in the diplotene or later stages characteristic of the oöcytes of amphibian females.

Of 56 *Amblystoma maculatum* reared in the laboratory in 1927 30 were females and 26 males. These numbers give an approximation of the expected 1 : 1 ratio. In this species, however, the majority of the transplants used were furnished by very young donors (stages 21 to 25 [1]) and but few gonads developed. In only two cases in which the sex of the donor was known to differ from that of the host was a gonad present in the graft. In one of these two the gonad was small and of the indifferent type, while in the second it was of a type combining features of both ovary and testis.

From the above it may be seen that relatively little evidence bearing upon the problem of sex-reversal was obtainable from grafts the donors of which had survived to sex-differentiation. But in several cases in which the donor had died before reaching this period, the transplant furnished by it was found to contain a gonad differing in sex type from that of the host in which the graft had developed. In these cases it would appear that donor and host must have been unlike in sex, but that the gonad of the graft had differentiated in a fashion determined by the organization of the transplant previous to its isolation from the donor. These cases may now be described in some detail.

No. 211.—Transplant from *A. jeffersonianum* of stage 29 implanted in *A. maculatum* of stage 25. The donor died 18 days after operation. The host, killed 61 days after operation, proved to be a female. A section of the ovary is shown in Fig. 1. The central ovarian cavity is well developed, and the germ cells are peripheral in position. Their nuclei are largely in the heterotypic prophase stages characteristic of the early urodele ovary, although few in number or lacking in the testis, as a rule, until a much later period of development. The graft removed from this host

[1] The stages referred to throughout this paper are those of Harrison's series of standard stages.

included a fairly large gonad of testicular type (see Fig. 2). No central cavity is present. The germ cells are somewhat uniformly scattered through the organ, intermingled with numerous smaller cells which constitute the 'sex cords' (anlagen of duct system), and the stromal and sustentacular elements of the testis. The germ cells are all in spermatogonial stages; heterotypic prophases are entirely lacking.

No. 284.—Transplant from *A. maculatum* of stage 30 implanted in host of same species and stage. The donor was killed by the host 37 days after operation. The host, autopsied 58 days after implantation of the graft, is a female. Although sex-differentiation had but recently occurred, the ovary has the characteristic central cavity and peripheral oöcytes with nuclei in heterotypic prophase (see Fig. 3). The gonad found in the transplant is a pear-shaped testis attached by a stalk to the surface of the graft mesonephros. It lacks the central cavity characteristic of the ovary, and shows the more uniform distribution of germ cells typical of the young testis (see Fig. 4). No heterotypic prophases are present, all germ cells being in spermatogonial stages.

No. 244.—Transplant from *A. jeffersonianum* of stage 31, implanted in host of same species and stage. Donor presumably devoured by host at about 31 days after operation. The host, autopsied 61 days after operation, is a male; a section of one testis is shown in Fig. 5. As is frequently the case in males at this stage of development, the testes of this animal show a few cells in the spermatocyte stage, but the germ cells are distributed in the fashion characteristic of the testis, and no central cavity is present. For comparison with the testis of the host a section of the gonad of the graft is shown in Fig. 6. This gonad must be interpreted as an ovary in an early stage of sex-differentiation. Although no central cavity is yet present, the germ cells are arranged in a layer around the periphery of the gonad and are for the most part oöcytes in earlier stages of the heterotypic prophase. By comparison of Fig. 6 with Figs. 1 and 3 (ovaries of fairly early stages of differentiation) it will be readily appreciated that this graft gonad is ovarian in nature. The differences between it and the graft gonads of Figs. 2 and 4 (testes) are

clearly evident from the photographs, and need no further comment.

The three cases above described show clearly that a gonad developing in a graft need not agree in sex type with the gonad of the host. It may be logically inferred that in these three cases the sex-differentiation of the graft gonad was determined by the organization in the implanted mesoderm previous to its removal from the donor embryo.

In a few cases in which both donor and host lived until after sex-differentiation and were found to be of unlike sex, a gonad was present in the graft. These cases, however, are less satisfactory than the preceding, since the gonad of the graft is either in an early stage of sex-differentiation or is of atypical structure. Three such cases will now be described.

No. 207.—Transplant from *A. jeffersonianum* of stage 29 implanted in *A. maculatum* of stage 25. The host, killed 61 days after operation, is unquestionably a male, although a few germ cells in heterotypic prophase are found in one of the testes. A section of the testis is shown in Fig. 7. The donor, a female, was not killed until 78 days after operation. The gonad shown in Fig. 8 is therefore more advanced in development than the testis of Fig. 7. The gonad found in the graft is small and in an early stage of sex-differentiation. Although no central cavity is present, the germ cells tend to take a peripheral position. Of the 95 germ cells present, 38 are in early stages of the heterotypic prophase. Considering all its structural features, this gonad should be classed as an ovary. In the peripheral arrangement of its germ cells, and in the high proportion of these cells found in heterotypic prophase, it is clearly similar to the gonad of the donor rather than to that of the host.

No. 190.—Transplant from *A. jeffersonianum* of stage 33 implanted in host embryo of same age and species. The host, killed 64 days after operation, is a female (see ovary in Fig. 10). The donor, killed at the same age as the host, is a male (see Fig. 11). The gonad of the graft is atypical in structure in that an unusual amount of stroma is present, in the form of a mucous type of connective tissue (Fig. 12). It may nevertheless be classed as testis rather than ovary. The germ cells, though frequently included in the covering epithelium, are predominantly

scattered through the central part of the organ. No central cavity is present. Sex cords (anlagen of duct system of testis) are recognizable as groups or strands of smaller cells, in some sections extending a third or more of the length of the gonad. The germ cells are for the most part spermatogonial in type, only three or four of the several dozen present being in heterotypic prophase, and none of these having the characteristics of growing oöcytes. Though of atypical structure, this gonad cannot be considered as undergoing transformation from testis into ovary. Aside from the abundance of mucous connective tissue, its structural features are clearly similar to those of the testis in the donor. Atypical gonads of the same general appearance may develop in grafts from a male donor implanted in a male host. The peculiarities of structure exhibited are therefore due, probably, to the action of local environmental factors rather than to the activity of sex hormones secreted by the gonads of the host.

No. 188.—Transplant from *A. jeffersonianum* of stage 33 implanted in host of same age and species. Both donor and host were killed 64 days after operation. The host is a female, the donor a male (see Figs. 13 and 14). The gonad of the graft is an atypical structure difficult to classify (see Fig. 15). Neither typical ovarian cavity nor testicular duct system is recognizable. The germ cells are predominantly peripheral in location, although frequently scattered or in masses deeper within the stroma. In one instance a mass of germ cells lies in a cavity, with no apparent attachment to other tissues of the gonad; these cells show marked degenerative changes. The cells at the periphery of the gonad frequently exhibit a grouping or 'nesting' comparable to that of young oöcytes in a normal ovary. Though for the most part in heterotypic prophase (several hundred such cells must be present) these germ cells seem never to progress beyond the pachytene stage. If the gonad were actually ovarian, some few at least of these cells might be expected to pass through the diplotene stage and then enlarge as growing oöcytes. This has been found to occur in those atypical gonads which have developed in grafts from female donors. In this gonad, however, no growing oöcytes are present, numerous pyknotic and fragmenting nuclei indicating the degeneration of the germ cells

during the pachytene stage rather than their continued development.

While it might appear at first glance that the features exhibited by this gonad have resulted from the action of the hormones of the host, it is highly probable that many of its peculiarities are referable to the growth potentialities of the implanted tissue as modified through local environmental influences. The donor furnishing this particular transplant exhibits an unusual number of spermatocytes in its one (left) gonad. Four such cells may be recognized in the section shown in Fig. 14 (at left). Presumably the tissue implanted possessed the potentiality for developing a gonad in which unusual numbers of heterotypic prophases would have appeared precociously, even without an endocrine stimulus from a female host. As to local environmental conditions, it may be noted that the gonad was attached by a very delicate fold of tissue, and was apparently poorly vascularized. The latter condition alone would be unfavorable to the development of a gonad of normal histological structure.

In addition to the graft gonad above described (No. 188) two other specimens exhibit features which might possibly be interpreted as modifications due to the action of sex-differentiating hormones. In one of these the graft gonad consists of a central core of testicular character overlaid by a cortex ovarian in type. This structure resembles the modified testes described by Burns ('28) as resulting from the action of ovarian hormones. The position of this particular graft in the body of the host, however, is such that some of the primordial germ cells of the host may actually have entered into the make-up of the graft gonad. If this be the case, this structure must be regarded as a 'mosaic' gonad derived from two preprimordia of unlike sex-potentialities rather than as a testis undergoing sex-reversal due to the endocrine influence of a female host. It is significant that graft gonads developing in sites sufficiently far ventral to exclude the possibility of actual contribution of host germ cells generally show no indication of sex-reversal (see Figs. 2, 4, and 6).

Among those cases in which only the host survived until the period of sex-differentiation are seven in which the gonad of the graft agrees in type with those of the host. While a reversal of sex in these few cases cannot be positively excluded, it is

rendered exceedingly improbable by the fact that in five other cases the gonad of the graft is of opposite sex from those of the host. Examples of this latter group have already been described (Nos. 211, 284, and 244; Figs. 1 to 6).

Discussion.

The outstanding feature of the results described in the preceding pages is the apparently independent sex-differentiation of the gonads which develop in grafts. Although in one or two cases such a gonad has been modified in a fashion suggesting an influence from sex hormones of the host, in no case is a complete early reversal of sex clearly indicated. So far as can be determined from cases in which the sex of the donor is known, the primary sex-differentiation in the gonad of the graft always proceeds in a fashion determined by the sex of the donor. In five cases in which the sex of the donor is not known, the gonad is of opposite sex from that of the host. In four of these cases, gonads with the features characteristic of a testis have differentiated in grafts implanted in female hosts, while in the fifth an ovary has developed in a graft implanted in a male.

It is difficult to reconcile these findings with the conclusions reached by Burns ('25) from his studies on the sex of parabiotic twins in *Amblystoma*. Burns finds that the sex of the two members of any pair is always the same. From this he is led to infer that complete reversal of sex has occurred in one member of all two-sexed pairs, such reversal being accomplished before sex differences in the gonads become morphologically distinguishable. He assumes that when embryos of unlike sex are joined in parabiosis there results a condition of close balance or unstable equilibrium, which is broken if one animal of the pair gains a slight advantage, presumably through earlier or more abundant output of sex-differentiating hormones. All hormones being mingled in the blood stream, and neither sex being constantly prepotent, either the male or the female hormone may become dominant. Such domination being established before the onset of morphological sex-differentiation, the phenomena of this period will be identical in the two members of any parabiotic combination, or essentially so. The twin which has undergone reversal thus differentiates directly without first exhibiting the

sexual characters to be expected from its genetic constitution. Under these conditions, a reversal cannot be detected by study of developmental stages of the gonads but must be inferred from the absence of two-sexed pairs after morphological differentiation has been completed, unless it be assumed that all such pairs have been eliminated through a selective mortality.

In discussing his results, Burns considers the possibility that a 'selective' mortality has operated to eliminate all heterogeneous (male-female) pairs, permitting only homogeneous pairs to survive. While this explanation cannot be positively rejected, Burns regards the occurrence of a selective mortality as highly improbable. Although the death rate among his operated animals is very high (about 77 per cent.), he believes that it is possible to explain it without postulating a physiological incompatibility of the sexes so profound as to induce the death of all two-sexed pairs. Witschi ('27) has demonstrated that no such incompatibility exists in the frog, since he finds the expected number of mixed pairs at metamorphosis of his parabiotic animals. If we assume that among Burns's experimental animals there was likewise no selective mortality eliminating mixed pairs, we are forced to conclude that parabiosis induces an early sex-reversal in one member of every two-sexed pairs.

If sex-reversal in parabiotic twins in *Amblystoma* be assumed to have occurred in the manner postulated by Burns, it would be logical to expect a reversal of sex in the gonad of a graft implanted in a host of opposite sex from that of the donor. The bulk of the transplant is small compared with the entire body of the host, and the gonad to which the graft gives rise is but a fraction of the size of the host's own gonads. Under these conditions there should exist no state of near-equilibrium as regards sex. If sex-differentiating hormones are produced previous to morphological sex-differentiation, those of the host should always, from their greater abundance, be able to dominate the differentiation of the gonad in the graft;[2] the latter, therefore, should always

[2] The gonad of the graft is often somewhat retarded in development as compared with those of the host, possibly, in some cases, because of inadequate nutrition. Such retardation of its development should favor modification of the graft gonad by the gonads of the host, assuming that sex differentiating hormones are poured into the circulation when the gonads reach a certain stage in their differentiation.

agree in type with the gonads of the host. Yet the gonad of a graft is clearly able to develop as ovary in a male host, or as testis in a female. In none of my animals could sex-reversal be demonstrated as having preceded the primary sex-differentiation.

Since the extent to which a hormone may modify an embryonic structure probably depends in part upon the period of development at which it is introduced and the time during which it is allowed to act, these conditioning factors may well be compared for parabiotic twin and graft.

In Burns's experiments, *Amblystoma* embryos were joined in parabiosis at about stage 28 of Harrison's series. In my own experiments many of the grafts were implanted at this or even earlier stages. In none of the cases considered in this paper was either donor or host more advanced in development than stage 34 at the time of operation. In neither the parabiotic twins at the time of union nor in the host receiving an implant has the blood yet begun circulation. While it is probable that the blood streams of embryos joined in parabiosis are in communication from the time the circulations of the two first become established, my observations indicate that the graft becomes vascularized at a correspondingly early period in its development. In short, the sex-modifying influence of the host upon the graft should be exerted fully as early as the influence of an embryo upon its parabiotic twin, assuming that this influence is mediated through the activity of substances transported by the blood.

As regards the actual time elapsing between operation and autopsy, the advantage appears to lie with the parabiotic twins. Burns states that among the pairs of his series even the best did not show sex-differentiation until seventy days, while the general average required considerably longer (eighty to ninety days) for sex to become clearly distinguishable. In my own animals sex was usually determinable without difficulty at fifty days after operation. The longer indifferent period in the parabiotic twins doubtless results chiefly from growth retardation due to difficulties in feeding. In any event, it greatly increases the period over which one animal is subjected to the influence of the other before morphological sex-differentiation occurs. Possibly in this prolonged indifferent period the physiological state of the gonads in one animal may be so altered through the influence

of its opposite-sexed twin that at the time morphological sex-differentiation finally occurs the gonads of the two animals differentiate in identical fashion. In my own experimental animals the shorter indifferent period may be insufficient to effect such a physiological reversal in the gonad of the graft, which in consequence differentiates as determined by the genetic constitution of the donor. In the parabiosis experiments of Witschi the indifferent period (in *Rana sylvatica*) is likewise short, which may possibly explain the fact that sex-reversal of the female follows rather than precedes the primary morphological differentiation of the gonad.

It is also possible that conditions attendant upon development of the graft may render it less subject to hormone influence from the host than is a parabiotic twin to the influence of its mate. Since the graft usually becomes well vascularized, however, it would seem that the nutritive materials and hormones of the host's blood stream should be as readily available for the gonad of the graft as for the host's own gonads. As has been stated before, sex-differentiating hormones of the host should be but little diluted by antagonistic hormones secreted in the graft. Moreover, the removal of the graft from its natural environment in the donor while in a germ-layer stage and its implantation into an essentially foreign situation should, if anything, disturb the action of local factors affecting sex-differentiation, and facilitate the modification of this process through hormones produced by the host. It would seem that in a graft the developing gonad has been removed from both the endocrine and environmental influence of the donor and subjected to the influence of the host in a far more complete fashion than the gonads of one parabiotic twin can be brought under the influence of the other embryo of the pair.

From comparison of the conditions acting upon parabiotic twin and graft, we may conclude that two, at least, possibly have significance in determining the difference in the results obtained. First, the greater time required for morphological sex-differentiation in parabiotic twins may permit an influence of one animal upon the other such as would not be possible in the case of a graft gonad differentiating in from half to two-thirds of the same period. Secondly, the fact that in one case (parabiosis)

the gonad has remained undisturbed in the organism, while in the other its preprimordium has been implanted in an ectopic situation in another individual, may possibly explain the different way in which it reacts preceding or during sex-differentiation.

The results obtained by the writer in *Amblystoma* are not without parallel from experimental work on other vertebrates. Willier ('27), from his study of the differentiation of chick gonads implanted in the chorio-allantoic membranes of either male or female hosts is led to the conclusion that "the course of sex-differentiation in the chick embryo is apparently not determined by the action of sexual hormones circulating in the blood stream." He believes that "hormonic sex-differentiating factors of the host embryo are either absent, or if present, they are ineffective in the modification of the engrafted sexual glands." Witschi ('27*b*) reaches similar conclusions from one of his latest studies on sex-differentiation in *Rana temporaria*. He finds that the implantation of a large graft of adult frog testis in tadpoles of this species does not "exert the least influence upon the larval and early post-larval development of the gonads." In both frog and chick, therefore, the indifferent gonads are found to undergo their primary sex-differentiation apparently unmodified by sex hormones from outside sources. In cattle, too, recent studies may be interpreted as showing that even when the chorions of two-sexed twins are fused at a very early period, the gonad of the female co-twin first begins to differentiate as an ovary, and only later undergoes modifications leading to the production of the characteristic free-martin gonad (Lillie, '23; Bissonnette, '28).

That the vertebrate ovary in situ may be modified in its development subsequent to its primary sex-differentiation is apparent from the studies of Lillie ('17) and others on the free-martin, or from the cases of sex-reversal in parabiotic frogs reported by Witschi ('27*a*). That these same gonads would have undergone a comparable modification if implanted as grafts in a host of the opposite sex has not been actually demonstrated. According to Willier, no modification of engrafted gonads of the chick is demonstrable after a period of nine days on the host embryo. It is conceivable, however, as Willier states, that the transplantation of the embryonic sexual glands into chicks after hatching might yield different results than when these same

glands are implanted on the membranes of embryonic hosts. Greenwood ('25) has reported the development of spermatic tubules in grafts of the left ovary taken from chicks two to four days after hatching and implanted in young chicks of the same age. It would appear probable, therefore, that isolation and implantation of a gonad (or its preprimordium) do not necessarily prevent the modification of that gonad through the action of sex-hormones of the host: *i.e.*, there remains possible an inhibition of growth, or an induction of growth, in those parts (as for example the medullary cords of the bird's ovary) which have retained their embryonic capacity to react in a specific fashion to growth stimuli.

The grafts described in this paper were in no case left implanted in the host for a period longer than seventy days. Although in none of the grafts recovered had the gonad undergone a complete reversal of sex previous to its primary differentiation, it is possible that in one or two cases it had undergone some slight modification which might be ascribed to the action of sex hormones of the host. Whether a complete reversal of sex might have occurred had the graft remained implanted for a longer period is problematic. From grafting experiments recently reported by Burns ('27) it is evident that sex-reversal in the gonads of *Amblystoma* is not complete even after periods of from fifty to seventy-six days in a host of the opposite sex. Burns transplanted gonads from larval stages, just before and just after the beginning of morphological sex-differentiation, into older larvæ in which sex-differentiation was more advanced. Since several of the grafts showed an admixture of the characteristics of the two sexes, it is possible that a complete reversal of sex might eventually have been effected.

Whether or not complete reversal of sex in *Amblystoma* may occur subsequent to morphological sex-differentiation, a reversal of sex preceding this period does not appear to be effected in gonads developed in grafts, when such grafts are implanted in an ectopic situation, such as the latero-ventral body wall. Whether implantation of the graft into its normal site would insure reversal of the gonad as postulated for animals joined in parabiosis still remains a question. The writer now has in progress an extensive series of experiments to test this point.

Summary and Conclusions.

1. An area of mesoderm which included the preprimordium of the gonad was transplanted from one *Amblystoma* embryo to another at stages 21 to 34. Such transplants, when taken from donors older than stage 25, gave rise to a gonad in a high percentage of cases. This gonad was ectopic in position, being attached to the inside of the lateral or ventral body wall, and was always far smaller than the normal gonads of the host.

2. Morphological sex-differentiation occurred in the grafts at from fifty to sixty days after implantation. All grafts were removed and fixed within seventy days. In several cases, gonads of testicular type were recovered from female hosts. In two cases gonads of ovarian type were found in grafts implanted in males.

3. In two cases in which donor and host were of opposite sex the gonad of the graft was modified in such fashion as to suggest an influence from sex hormones of the host. In no case, however, was the sex of the graft gonad completely reversed previous to the period of morphological sex-differentiation.

4. It may be concluded that gonads developed in ectopic grafts of the gonadal preprimordia undergo their primary morphological sex-differentiation according to the organization of the graft at the time of its removal from the donor.

5. If sex-differentiating hormones are produced by the host previous to morphological sex-differentiation, they are apparently incapable of bringing about reversal in the gonad of the graft. The possibility of reversal at a later stage of development is not excluded, since no grafts were allowed to develop for periods longer than seventy days.

6. The failure of the gonad in a graft to undergo sex-reversal previous to its morphological differentiation is in marked contrast to the complete reversal which appears to occur in parabiosis (cf. Burns '25).[3]

[3] Studies completed while this paper was in press indicate that the graft ovaries of Figs. 6 and 9 possibly owe certain features of their structure to the action of the testicular hormones of the host. These studies show that the developing ovary is readily modified if subjected to the continued influence of a testis resident in the same host, and that one of the first perceptible indications of this modification is the absence of the characteristic central ovarian cavity. These studies will be reported in a separate paper.

BIBLIOGRAPHY.

Bissonnette, T. H.

'28 Notes on a 32 Millimeter Freemartin. BIOL. BULL., Vol. 54, pp. 238–253.

Burns, R. K.

'25 The Sex of Parabiotic Twins in Amphibia. Jour. Exp. Zoöl., Vol. 42, pp. 31–89.

'27 Some Results of the Transplantation of Larval Gonads in Urodele Amphibians. Anat. Rec., Vol. 37, p. 163.

Greenwood, A. W.

'25 Gonad Grafts in the Fowl. Brit. Jour. Exp. Biol., Vol. 2, pp. 469–492.

Humphrey, R. R.

'27 The Fate of the Primordial Germ Cells of *Amblystoma* in Grafts Implanted in the Somatopleure of Other Embryos. Anat. Rec., Vol. 35, pp. 40–41.

Lillie, F. R.

'17 The Free-martin; a Study of the Action of Sex-hormones in the Foetal Life of Cattle. Jour. Exp. Zoöl., Vol. 23, pp. 371–452.

'23 Supplementary Notes on Twins in Cattle. BIOL. BULL., Vol. 44, pp. 47–77.

Willier, B. H.

'27 The Specificity of Sex, of Organization, and of Differentiation of Embryonic Chick Gonads as Shown by Grafting Experiments. Jour. Exp. Zoöl., Vol. 46, pp. 409–465.

Witschi, Emil.

'27*a* Sex-Reversal in Parabiotic Twins of the American Wood-Frog. BIOL. BULL., Vol. 52, pp. 137–146.

'27*b* Testis Grafting in Tadpoles of *Rana temporaria* L. and its Bearing on the Hormone Theory of Sex Determination. Jour. Exp. Zoöl., Vol. 47, pp. 269–294.

PLATE I. EXPLANATION OF FIGURES.

All figures on this plate are photomicrographs. Magnification 145 ×.

FIG. 1. Ovary of host No. 211, *Amblystoma maculatum*, killed 61 days after implantation of graft at stage 29. The central ovarian cavity is well developed, and the germ cells peripheral to it are chiefly oöcytes in heterotypic prophase. Compare with Fig. 2.

FIG. 2. Testis of graft recovered from host No. 211. Note the absence of a central cavity. The germ cells are uniformly distributed, and none are in heterotypic prophase. Compare with the ovary of the host (Fig. 1). This testis was attached to the body wall by a slender stalk not included in this section.

FIG. 3. Ovary of host No. 284, *Amblystoma maculatum*, killed 50 days after implantation of graft at stage 30. Ovarian cavity, peripheral arrangement of germ cells, and abundance of heterotypic prophase stages, as in Fig. 1. Compare with graft gonad of Fig. 4.

FIG. 4. Testis of graft recovered from host No. 284. Note absence of central cavity and heterotypic prophases, and the uniform distribution of the germ cells. Compare with the ovary of the host in which this testis developed (Fig. 3). The slender stalk attaching the testis to the mesonephros of the graft is not included in this section.

FIG. 5. Testis of host No. 244, *Amblystoma jeffersonianum*, autopsied 61 days after implantation of graft at stage 31. This gonad exhibits the scattered arrangement of germ cells and the absence of a central cavity noted in the testes of Figs. 2 and 4. Compare with graft gonad shown in Fig. 6.

FIG. 6. Ovary of graft recovered from host No. 244. Although the central cavity is not yet developed, the germ cells are peripheral in position and are for the most part in heterotypic prophase. This gonad thus resembles an ovary (see Figs. 1 and 3) rather than the testes of the host from which it was recovered (see Fig. 5).

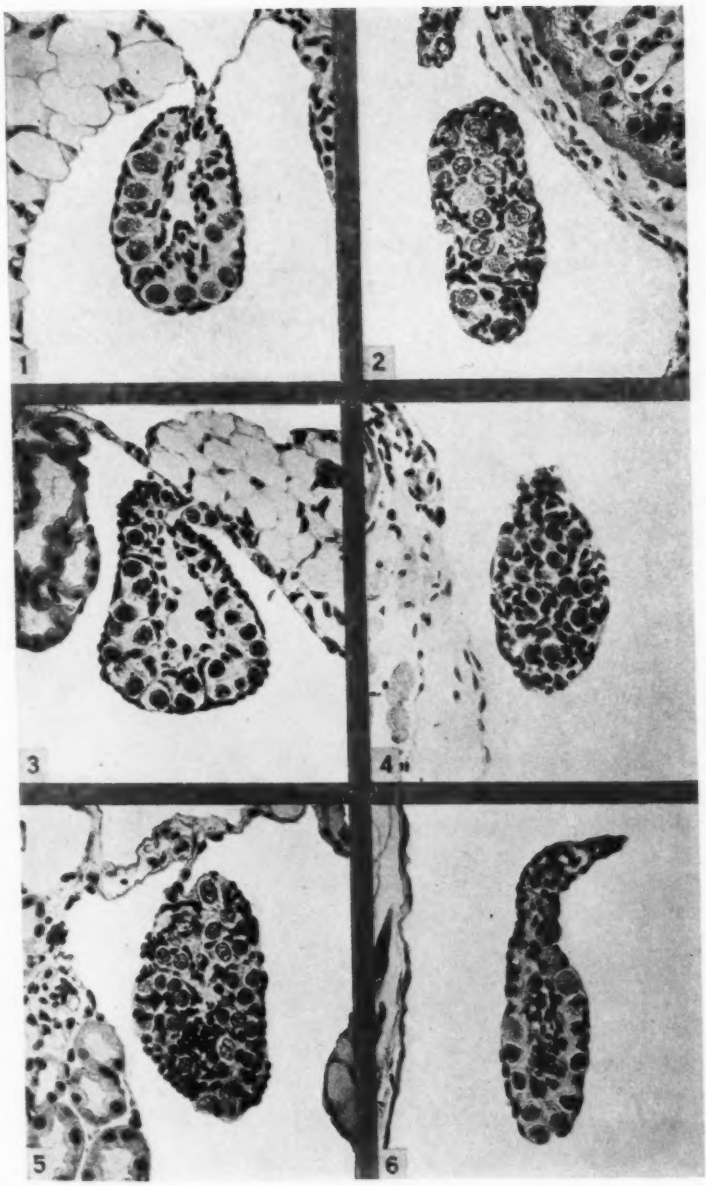

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PLATE II. EXPLANATION OF FIGURES.

All figures on this plate are photomicrographs. The magnification is 121 × except for Figs. 8 and 9, in which it is 162 ×.

FIG. 7. Testis of host No. 207, *Amblystoma maculatum*, killed 61 days after implantation of graft at stage 25.

FIG. 8. Left ovary of donor No. 207, *Amblystoma jeffersonianum*, killed 78 days after furnishing graft (at stage 29) for implantation in host No. 207. Due to the age at which this animal was killed, the ovary is advanced in development as compared with those of Figs. 1 and 3.

FIG. 9. Gonad of graft recovered from host No. 207. Though retarded in its differentiation, this gonad is apparently an ovary, since its germ cells are peripheral in arrangement, and a large proportion of them are in heterotypic prophase stages. Compare with Figs. 7 and 8.

FIG. 10. Ovary of host No. 190, *Amblystoma jeffersonianum*, killed 64 days after implantation of graft at stage 33.

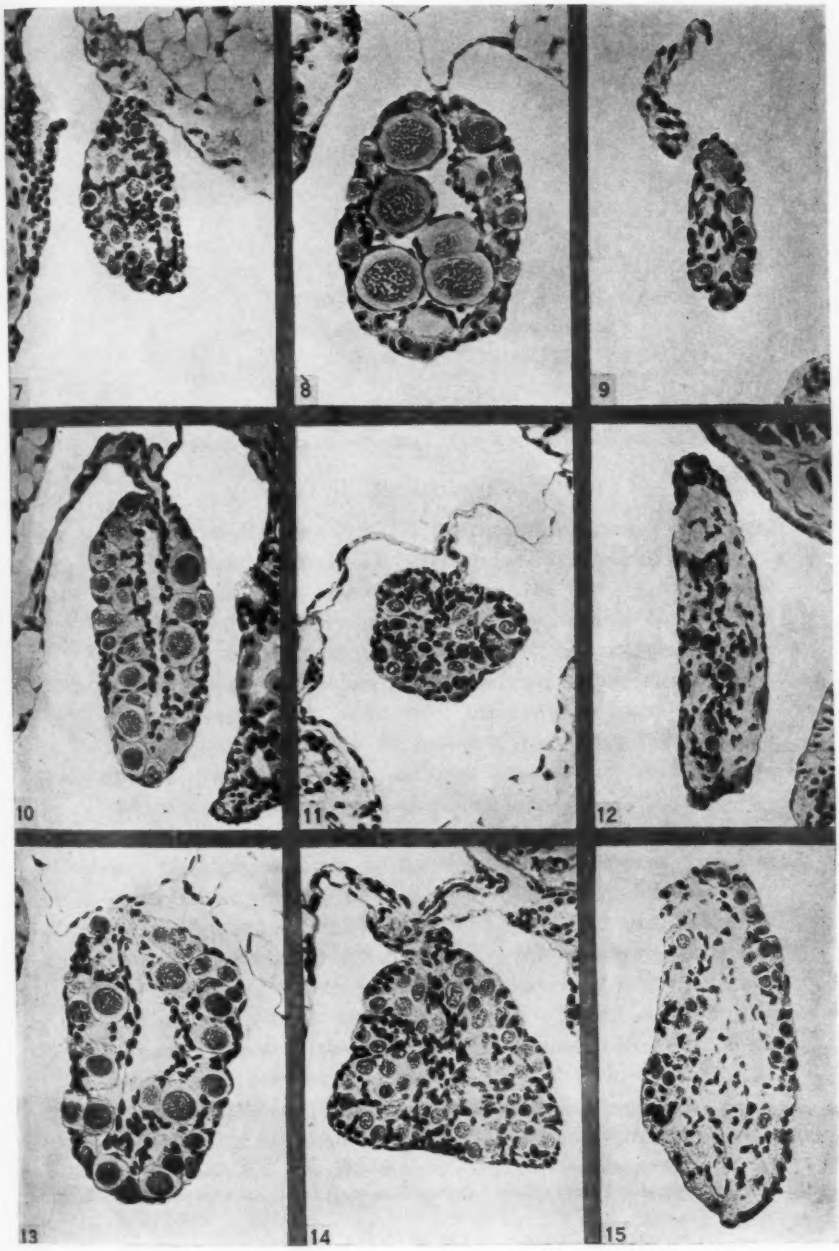
FIG. 11. Left testis of donor No. 190, *Amblystoma jeffersonianum*, killed 64 days after furnishing graft for implantation in host No. 190.

FIG. 12. Gonad of graft recovered from host No. 190. Though atypical in structure, due to the presence of mucous connective tissue, this gonad is apparently a testis. No central cavity is present, the germ cells are scattered, and but very few of them are in heterotypic prophase. Compare with gonad of donor (Fig. 11).

FIG. 13. Ovary of host No. 188, *Amblystoma jeffersonianum*, killed 64 days after implantation of graft at stage 33.

FIG. 14. Left testis of donor No. 188, *Amblystoma jeffersonianum*, killed 64 days after furnishing graft for implantation in host No. 188.

FIG. 15. Gonad of graft recovered from host No. 188. It lacks a central cavity, but has its germ cells predominantly peripheral in position, and frequently in groups or "nests" as in the ovary. Although many of its germ cells are in heterotypic prophase, this is true also of the testis of the donor. This gonad is possibly a testis modified by reason of its development in a graft in a female host.



R. R. HUMPHREY.

ON THE PROPERTIES OF THE GONADS AS CONTROLLERS OF SOMATIC AND PSYCHICAL CHARACTERISTICS.

XI. Hormone Production in the Normal Testes, Cryptorchid Testes and Non-living Testis Grafts as Indicated by the Spermatozoön Motility Test.[1]

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I. Introduction.

Advances in the study of the internal secretions are very often largely dependent upon the development of successful indicators for the substances concerned. Since the studies of Brown-Sequard, innumerable attempts have been made to increase our knowledge of the internal secretions of the sex glands, and indeed vast stores of information have been accumulated through these investigations. The chief difficulty in many of these attempts and especially in attempts to obtain the hormone principle in extractions, has been the lack of an applicable indicator of the substances sought for isolation.

A tremendous step forward in the study of the female hormone was the demonstration of the details of the œstrous cycle as indicated by vaginal smears first by Stockard and Papanicolau ('17) in the guinea pig and later by Long and Evans ('22) in the rat. By the vaginal smear method, one is enabled to determine the presence or absence of substances concerned with the regulation of the œstrous cycle. With such a useful indicator, the advances made in the study of the internal secretions of the ovary have been indeed marked.

On the male side, however, the situation has been a less happy one from the standpoint of real advancement. In some species

[1] This investigation has been aided by a grant from the Committee for research in problems of sex of the National Research Council; grant administered by Prof. F. R. Lillie.

of the bird, notably certain breeds of the domestic fowl, the male feather pattern, behavior, and head furnishings have afforded a good criterion of testicular presence and activity though many details were insufficiently known until of late to make conditions as well understood as was needed. The extensive work of Domm ('27) on the brown leghorn breed has given a much greater appreciation of the many pitfalls that present themselves in this field (for a review of the extensive literature on this subject, see Domm's paper).

When we approach the study of the internal secretions of the gonads in the male mammal, however, a careful analysis will show the marked absence of useful criteria to indicate the activity of the testis hormone, operating over limited periods of time. It is true that there is the sex impulse, supposedly entirely under the control of the internal secretions of the testicles, but many things lead us to believe this supposition to be erroneous. A castrated male theoretically should lose its attraction for the female, but I have repeatedly utilized guinea pigs castrated at 30 days of age as testers for the period of female acceptance for some months after castration. Stone ('27) has recently reported that young male rats castrated at the age of three months will continue to copulate with females for periods of four, five and even eight months. And it is reported that the eunuch, though castrated early in life, will years afterward have not only an attraction toward the female but experiences a degree of satisfaction in this association.

It is likewise true that the growth of the penis, seminal vesicles (when present), prostate, etc., are to a large extent dependent upon the internal secretions of the testis but not only have the variations in such structures proven so great as to make an assay of a given experimental procedure difficult and often impossible, but also, if castrations are made on adult animals, to be followed by such procedures as testis transplantations, injections or other possible approaches, the question of the condition of these structures as representing a balance between postoperative regression or possible stimulation from the materials or conditions utilized often presents insurmountable barriers.

Such other indicators for testicular internal secretions as individual body weight, body length, fat deposition, hair coat,

and pugnacity as have been utilized by other workers serve often to mislead the investigator due to the lack of specificity of the indicator (for further criticisms of this phase see my papers, '21 and '22).

During the course of a study of the physiology of the scrotum or its heat regulating effects on the generative tissues of the testis (Moore, '24*a*, *b*; '26, '27, and '28; Moore and Quick, '24) a possible, fairly satisfactory indicator for the internal secretions of the testis in the differential survival of the capacity for motility of epididymal spermatozoa was discovered by accident. The same conditions were also discovered by Benoit ('26) a little earlier, in the course of his beautiful work on the histology and cytology of the epididymis. The application of this "spermatozoön-motility" test for the testis hormone has been under investigation in this laboratory for longer than three years. The principle of the test may be expressed in details for the guinea pig.

When both testes of an adult guinea pig are removed from the animal, leaving the inferior portion of the epididymides, containing their millions of spermatozoa, in the normal scrotal position, one finds that the spermatozoa gradually lose their capacity for motility when these are suspended in physiological saline solution. A lessened capacity for motility is evident within a few days after testis removal and seldom can one see any degree of motility in the spermatozoa after a period of twenty-three days following the operation. However, if instead of removing both testes, one is allowed to remain normal, the single, opposite, epididymis will contain spermatozoa that show motility when suspended in saline solution for a period of sixty-five to seventy days (Moore, '28). The difference between the 23 days retention of the capacity for motility when both testes have been removed and that of 65 days when one testis has remained, has been proven to be an expression of the internal secretion of the testis (for further details of this reaction see Moore, '28).

While it is freely admitted that the spermatozoön motility reaction has many limitations we have found it very useful and it will continue to be useful until a better hormone indicator has

been discovered. In the following pages a few items of information with respect to its usefulness will be presented.

II. Hormone Production by Normal Testes.

Utilizing the spermatozoön motility reaction in the guinea pig as described above, I have attempted to study hormone production in the normal testis to learn more concerning its action upon the life of spermatozoa when it is removed from the animal through castration at varying periods during the possible life of the mature germ cell. It has been indicated, for example, that under the influence of the full hormone compliment of one testis, the spermatozoön life, as shown by its capacity to exhibit motion in physiological saline solution, gradually wanes until after approximately 65 to 70 days it no longer responds to this stimulus. Should we, for example, wish to supply the hormone by testis transplantation or by injection of material supposed to contain it, we should be able, if possible, to test the effect in the shortest time within which the reaction will indicate any effect. What influence, therefore, does hormone supplied by a testis *in situ* exert when it acts for ten, fifteen or twenty days? The following procedure will present the method employed.

Young adult guinea pigs are operated under ether anæsthesia through a low mid-abdominal incision and one testis withdrawn into the field of operation. The testis is carefully separated from the inferior pole of the epididymis (tail portion), the internal spermatic vessels are ligated and the testis, along with the head and body of the epididymis removed. The remaining (inferior) portion of the epididymis, connected with its vas deferens, is then carefully replaced in the scrotum—a necessary precaution (see Heller, '29). Ten or fifteen days later the opposite normal testis is removed entire through a scrotal incision. At selected intervals after the second operation, the animals are sacrificed, the isolated epididymis finely hashed with scissors in a small quantity of physiological saline and examined immediately with the microscope for spermatozoön motility. To properly express gradations in motility * signs have been employed in which the normal movement is expressed by ****; the barest vibratile movement on the part of a few spermatozoa (perhaps 1 in 10,000 will contract weakly with little or no trans-

lation) is rated *. Where no movement can be detected the observation is designated 0.

Table I. will serve to illustrate the observations on the motility of spermatozoa obtained from the isolated epididymis of animals whose normal testis was allowed to remain for 10, 15, 20, 30, and 40 days after the epididymis to be tested had been isolated.

TABLE I.

UNILATERAL EPIDIDYMAL ISOLATION; OPPOSITE TESTIS REMOVED SUBSEQUENT TO ISOLATION AS INDICATED IN DIFFERENT GROUPS (GUINEA PIG).

Animal.	Epididymal Isolation.	Killed.	Days after Isolation.	Motility.	Excess Life beyond 23 Days Attributed to Hormone Effect.
			(delayed removal 10 days)		
517	1–31	3–1	30	*	7 days
518	1–31	3–3	32	*	9 days
520	1–31	3–5	34	0	
521	1–31	3–5	34	0	
			(delayed removal 15 days)		
406	6–10	7–11	31	**	8 days
407	6–10	7–14	34	***	11 days
409	6–10	7–20	40	0	
409	6–10	7–20	40	**	17 days
410	6–1	7–24	44	**	20 days
411	6–11	7–29	48	0	
412	6–11	7–29	48	0	
413	6–11	7–29	48	0	
414	6–11	7–29	48	0	
415	6–11	7–29	48	*	25 days
			(delayed removal 20 days)		
441	10–21	11–29	39	*	16 days
442	10–21	11–29	39	*	16 days
443	10–21	12–3	43	*	20 days
444	10–21	12–3	43	*	20 days
445	10–21	12–6	46	0	
446	10–21	12–6	46	0	
			(delayed removal 30 days)		
450	10–25	12–8	44	**	21 days
447	10–25	12–17	5	*	30 days
451	10–25	12–20	56	*	33 days
452	10–25	12–24	60	0	
			(delayed removal 40 days)		
469	12–3	1–25	53	*	30 days
472	12–3	1–25	53	0	
470	12–3	1–27	55	0	
473	12–3	1–27	55	*	32 days
474	12–3	1–31	59	0	

To understand the observations recorded it must be remembered that when an epididymis is isolated from its testis and both testes are removed from the animal, the spermatozoa contained within an epididymis retain their capacity to show motility for a period of 23 days; this we may call the basic life period and realize that they will live for this period without any hormone being produced by the testicle.

From the table it can be seen that under "delayed removal 10 days" the spermatozoa were observed to show motility for 32 days or nine days longer than expected, had both testes been removed at the time of epididymal isolation. We see, therefore, that the hormone supplied by the normal testicle for a period of ten days before its removal, actually extended the life of the spermatozoön nine days. Similarly, hormone supplied for fifteen days extended the retention of the capacity for motility for a similar length of time (actually slightly longer since in animal 415 a few sperm were seen to move slightly on the 48th day after isolation or an increase of 25 days above the basic 23 days expected). When the normal testis was allowed to remain 20 days after epididymal isolation, motile capacity was increased 20 days beyond what it would have been had both testes been removed at the first operation. Hormone supplied by the normal testis for 30 days permitted retention of spermatozoön motility up to 56 days or 33 days longer than the natural life without hormone being supplied. A hormone supply from the normal testis for forty days increased the sperm life by little more than thirty days. But it must be remembered that as we add to the length of time after operation we gradually approach the natural limits of spermatozoön life even with a full compliment of hormone; this limit is 65 to 70 days. We could not therefore expect the relative progressive effectiveness to continue much beyond a 40-day normal testis retention because of the approach to the maximum period of persistence of sperm under a continuous hormone influence. In an earlier paper I have emphasized that even utilizing the greatest possible care in the selection of standard animals for operation and in doing the operation itself, there is an individual animal variability that cannot be eliminated; at best we can only make an approach toward quantitative relationships.

Utilizing the basic 23 days as the approximate maximum of retention of the capacity for motility on the part of spermatozoa when no hormone is being supplied (and in scores of observations I have never observed motility for periods above 23 days) we see that one can actually detect the influence of the testis hormone when it is supplied for only ten days. Due to the individual animal variability, I would consider attempts to read reactions more finely as decidedly unprofitable. In fact, to err on the safe side, I have arbitrarily chosen to regard any tested substance or condition involving gonads as negative unless the capacity for motility is retained for thirty days or longer.

It is of interest to examine the data of the above table with reference to what they may tell us of hormone production and storage. When a ten day hormone supply by the normal testis shows an effect of ten days in the reaction, and likewise when spermatozoön motility is extended 15, 20, and 30 days beyond the basic expectations in conditions wherein the testis was present for 15, 20 and 30 days after epididymal isolation, one must conclude, I believe—(1) that hormone secretion is a continuous process and (2) that the hormone is not stored within the body. When hormone is supplied by the normal testis, for 10 days, the reaction indicates an effect for the same length of time.

III. Hormone Production in Cryptorchid Testes.

It has long been known that man or the domestic mammals may experience a failure of testicular descent into the scrotum. Such animals, although always sterile, nevertheless possess their full compliment of secondary sex characters; they are spoken of as Cryptorchid individuals. The undescended testes of such animals have long been known to lack a germinal epithelium; the gametogenetic function of the testis is deficient but its internal secretory effects are not visibly diminished. It is now known that a normal testis removed from the scrotum and confined within the abdomen very rapidly loses its germinal epithelium and assumes within a month or two, almost identical characteristics to those testes that have never descended. The cause of the degeneration of testes confined within the abdomen has been found to be the warmer environment of the abdomen

and the function of the scrotum has thus been seen to be that of a local thermoregulator (for details of this work see Moore, 1924*a*, 1924*b*, and 1926; Moore and Quick, '24).

Regarding the amount of hormone produced by such a cryptorchid testis, occurring normally or artificially made, little is known. It could be assumed perhaps that a smaller quantity of hormone might be required to produce or to maintain the secondary sex characteristics than would be required to maintain completely normal male conditions. Lipschutz and his co-workers have maintained that in the rabbit 1 per cent. of the normal amount of testicular tissue is sufficient to maintain all the secondary sex characters ('22). On the other hand, it has been assumed by some investigators that any condition leading to an "Apparent increase in interstitial cells" whether by testis transplantation, X-rays, vasoligation (this latter is the basis of the contentions underlying the ideas of the Steinach rejuvenation hypothesis) or any other means, presages an increased production of hormone. The argument proceeds from the assumption—First, that the apparent compensatory hypertrophy of interstitial cells is real (see discussion Moore, '24*a*; Bascom, '25); and second, that the hormone is produced exclusively by the Leydig cells. Some authors have gone so far as to speak of castrated males, bearing testis grafts containing appreciable amounts of interstitial tissue, as "supermales," an implication that I consider without any basis of fact.

In order to gain any new information possible regarding the quantitative aspects of hormone production by cryptorchid testes, I have utilized the guinea pig in the following manner: Young adult guinea pigs have been operated so that one testicle was removed from the scrotum into the abdomen and the inguinal canal closed to prevent scrotal redescent. Four months, and five months, later a second operation was made to isolate the normal epididymis from, and to remove, the normal testicle. We thus have an isolated epididymis with its spermatozoön content to use as a test for the hormone produced by the opposite degenerate four or five months experimental cryptorchid testis.

Table II. is a record of observations made on eleven animals in which one testis was confined to the abdomen for four months

and upon six animals where hormone supply came from a testis confined in the abdomen for five months.

TABLE II.

UNILATERAL CRYPORCHIDISM FOUR MONTHS; NORMAL TESTIS REMOVED FROM EPIDIDYMIS, AND EFFECT OF FOUR MONTHS CRYPTORCHID TESTIS ON SPERMATOZOÖN MOTILITY DETERMINED.

Animal.	Isolation of Normal Epididymis.	Killed.	Days since Isolation.	Motility.	Wt. of Cryptorchid Testes.*
393	Oct. 13	Nov. 25	43	***	Not recorded
394	Oct. 13	Dec. 5	53	**	0.26 gms.
402	Oct. 17	Dec. 8	52	***	0.424 "
396	Oct. 13	Dec. 12	60	*	0.21 "
397	Oct. 13	Dec. 12	60	0	0.175 "
398	Oct. 13	Dec. 17	65	*	0.158 "
400	Oct. 13	Dec. 17	65	0	0.130 "
403	Oct. 17	Dec. 21	65	*	Not recorded
404	Oct. 17	Dec. 26	70	*	0.170 gms.
416	Oct. 17	Dec. 31	75	*	0.255 "
417	Oct. 17	Jan. 7	82	0	0.205 "
	UNILATERAL CRYPTORCHID FIVE MONTHS; SUBSEQUENT TREATMENT SIMILAR TO ABOVE.				
475	Apr. 28	June 26	59	**	0.153 gms.
476	Apr. 28	June 30	63	*	0.13 "
477	Apr. 28	July 6	69	0	Not recorded
478	Apr. 28	July 6	69	*	Not recorded
479	Apr. 30	July 12	73	0	Not recorded
481	Apr. 30	July 12	73	*	0.095 gms.

* Testis weight, without epididymis or fat body.

It will be seen from Table II. that spermatozoa within the isolated epididymis have been observed to retain their capacity to show movement on proper stimulation for seventy to seventy-five days. Since the normal testicle, actively carrying on its spermatogenetic activity supplies only sufficient hormone to permit the sperm to live for the same length of time, we must conclude that the hormone producing capacity of a non-gametogenetic, degenerative, or cryptorchid testicle as measured by the spermatozoön motility test is the equivalent of the normal testis.

Figure 1 is introduced to show the histological character of the testicle after abdominal confinement (experimental cryp-

torchidism) for a period of seven months; the microphotograph shows a portion of a section from the testicle of animal No. 481. This testicle had been confined within the abdomen for a period of five months, when the epididymis of the opposite testicle was isolated for the subsequent spermatozoön-motility test and the normal testicle removed from the animal. Reference to Table II.

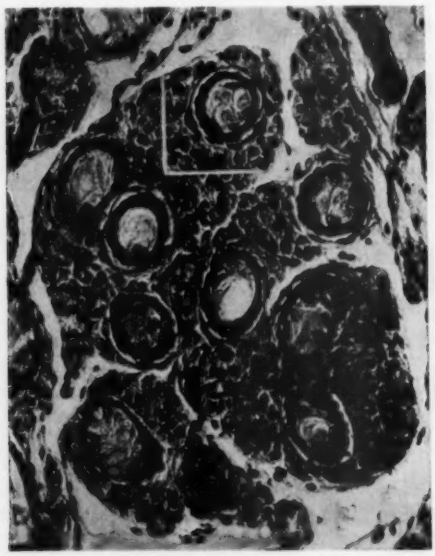

FIG. 1. Photomicrograph of portion of 7 months cryptorchid testicle (animal no. 481) showing shrunken seminiferous tubules separated by interstitial tissue.

will serve to recall that the test epididymis contained a few living spermatozoa 73 days after epididymal isolation and these few exhibited very weak motility on suspension in saline solution. When the animal was sacrificed on the 73d day after epididymal isolation the testicle had been confined in the abdomen for a period slightly longer than seven months. The weight of the organ, after removal of its attached epididymis, was 0.095 grams. Since the average weight of eight normal testicles, without the epididymis, removed from similar sized animals and at the same time of the year, was 1.7 grams (1.34 minimum wt.—

2.06 maximum) it will be appreciated that the weight of this cryptorchid testis represents 2.8 per cent. of the total testicular weight of the normal animal. Had the epididymides been included in this weight the percentage of the normal testicular weight represented by this cryptorchid testis would have been considerably less; the spermatozoön and secretion mass within the normal epididymis being very much greater than the slight fluid content of the cryptorchid epididymis. It can be concluded, therefore, that the cryptorchid testis representing 2.8 per cent. of the normal testicular mass was producing sufficient hormone to maintain the life of spermatozoa in the isolated epididymis for the same period as would the hormone produced by two normal testes.

Figure 1 shows that the seminiferous tubules of the seven month cryptorchid testis were very much reduced in caliber and consisted of a basement membrane, somewhat thickened, and a few Sertoli nuclei and reticulum; the tubules were rather widely separated by interstitial tissue. Fig. 2 is a drawing of the

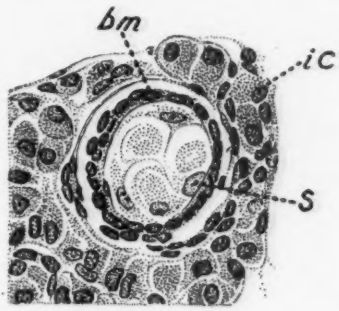

FIG. 2. Drawing of tubule marked off by white lines in Fig. 2. *bm*, basement membrane; *ic*, interstitial cell; *S*, Sertoli nucleus.

tubule marked off by white lines at the upper part of Fig. 1. The thickened basement membrane is more clearly shown and the character of the contents of the tubule indicates an absence of any germ cells; the nuclei that are visible are believed to be Sertoli nuclei. Careful microscopic study has failed to bring to my attention any cell that appeared different from those represented in this figure and it is for that reason that I believe no

germinal cells were present in this testis, at the time of its removal.

IV. Hormone Production by Testis Grafts.

The question of the function of testis transplants must of necessity be considered under at least two categories: (1) The function of grafts which have become successfully incorporated into the host organism and remain as living masses of testicular tissue, and (2) the function of such masses of testis tissue transplanted into various parts of the host organism, which by reason of host resistance to the transplant or because of too great a mass of tissue for vascularization, dies and is resorbed or sloughed out of the incorporation bed often with pronounced suppuration. It is known that living testis grafts can be obtained and that they will function. This question, along with the presentation of personal observations, has been reviewed by me at some length (Moore, '26). The question of the function of testis tissue transplanted into a host organism under conditions that have been so unfavorable as to prevent its retention and growth has been dealt with most usually by the clinician. For various reasons many cases of transplantation in man of human testis tissue or testicular tissue from another mammal such as the ram, boar, monkey or deer, have been done. The effects reported are so all-embracing that discredit of all effect is engendered (for discussion of this work see Moore, '26). In general it may be repeated that the effects reported have been expressed in terms of the subjective feelings of the patient—whether he may feel better after remaining quietly in bed for a week or longer after the operation; or whether after the suggestions and discussion of the question and the anticipations of the operation and its outcome, he has a greater desire for coitus; or whether the patient feels that he can walk more sprightly or feels that he can climb a stairs two steps at a time instead of the customary pre-operation one step. In short, such evidence is worthless from the scientific point of view.

In order to study by objective means the question of the function of such non-living testis transplants, I have utilized the guinea pig as the experimental animal and the spermatozoön motility test as an indicator of effectiveness. In an earlier paper

(Moore, '28) I included a few observations then at hand and have since given additional attention to the problem. The method employed, in brief, is the bilateral isolation of the epididymides along with removal of the testes from the animal. The two testes removed were replaced immediately in an especially prepared subcutaneous incorporation bed made by tunneling under the skin, with some destruction of skin musculature and a general scarification of the particular region. Each testicle, cut into two parts, was placed in a separate implantation bed, one on each side of the mid-ventral line of incision.

In addition to the subcutaneous transplantation of the animal's own two testes at the time of epididymal isolation, a few cases of multiple transplantations were studied with the idea that perhaps a small amount of hormone might be liberated from the introduced tissue which if introduced more than once would conceivably show a greater effectiveness. Accordingly at the time of bilateral epididymal isolation, two, one-fourth testes, were introduced subcutaneously at the time of the first operation as well as on the 3d and 5th day following. In each animal, therefore, six transplantations were made, the aggregate amount of tissue transplanted being one and one half testes. The observations on four such experiments are recorded in Table III*B*.

Since an arbitrary choice of thirty days after operation for effectiveness to be registered was made, animals were sacrificed close to this period for the study of the spermatozoön content of the isolated epididymides. From section II. it will be seen that motility of spermatozoa at this time would indicate the equivalent of effectiveness of the normal testis present for seven to ten days. Too much uncertainty surrounds the application of this test to make it profitable to attempt readings at an earlier date.

Table III. presents some of the observations recorded.

Among the nineteen animals whose isolated epididymides were studied for spermatozoön movement between the 25th and 36th day after autoplastic transplantation of two testes, only two animals have shown any movement of spermatozoa and in each case (animals No. 320, No. 456) observed on the 31st day the motility was the weakest possible for a positive reading. Amid

TABLE III.

A. THE EFFECT OF NON-LIVING TESTIS GRAFTS ON SPERMATOZOÖN MOTILITY (GUINEA PIG).

Animal.	Date—Operation.	Date—Killed.	Days after Operation.	Motility.
364	4– 6–27	5–12–27	36	0 many non-mot. sperm
365	4– 6–27	5–12–27	36	0
370	4– 8–27	5–12–27	34	0 " " " "
371	4– 8–27	5–12–27	34	0 " " " "
372	4– 8–27	5–12–27	34	0 " " " "
373	4– 8–27	5–12–27	34	0 " " " "
320	11–22–26	12–23–26	31	* (1 in 1000 weak mot.)
436	10–15–27	11–15–27	31	* (1 in 1000 weak mot.)
435	10–15–27	11–15–27	31	0
347	3– 8–27	4– 7–27	30	0 many non-mot. sperm
376	4–15–27	5–15–27	30	0 " " " "
377	4–15–27	5–15–27	30	0 " " " "
439	10–20–27	11–19–27	30	0 " " " "
440	10–20–27	11–19–27	30	0 " " " "
437	10–20–27	11–19–27	30	0 " " " "
455	10–26–27	11–25–27	30	0 " " " "
457	10–27–27	11–25–27	29	0 " " " "
458	10–27–27	11–25–27	29	0 " " " "
453	10–25–27	11–19–27	25	0 " " " "

B. MULTIPLE GRAFTS AFTER BILATERAL EPIDIDYMAL ISOLATION.

Animal.	Transplantation Days after Epididymal Isolation.	Killed, Days after Testis Removal.	Motility.
553	1st, 3d, 5th	30	0
554	1st, 3d, 5th	30	0
555	1st, 3d, 5th	30	*
549	1st, 3d, 5th	32	0

the field of millions of spermatozoa, here and there, an individual cell could be seen to show a weak contraction, perhaps a weak vibratile movement every thirty seconds; a rough estimate of 1 in 1000 was made to give a relative notion of the quantity of spermatozoa capable of movement. In contrast to this, eleven animals observed a shorter period of time after operation (25 to 30 days) were all negative; no spermatozoön movement could be seen. One animal (No. 555) receiving six transplantations of one fourth of one testis at three different operations subsequent to testis removal, showed a few spermatozoa capable of weak movement on the 30th day after operation, whereas

two others on the same day and one on the 32d day after operation failed to show any spermatozoa capable of exhibiting movement despite the fact that quantities of normal looking sperm were present.

These results show, therefore, that subcutaneous transplantation of testes provided such a small amount of hormone (if any at all) that its effect was less than the effect of a normal testis remaining *in situ* for seven to ten days after operation.

It is difficult or impossible, as pointed out above, to prevent individual animal variation. Whether the two positive readings on the 31st day are to be explained on this basis of more virile spermatozoa or as an indicator of some hormone effect cannot be stated. But since all operations were done alike, and equivalent masses of tissues transplanted, it would seem as if animals sacrificed earlier (between 25 and 30 days) would have given as strong or a stronger reaction than these three. In any event, should we attribute the results to hormone production and express it as a positive effect of the transplantations, the mildness of the reaction would still be evident. At best it is a questionable indication of hormone production.

The transplanted tissue reactions have been characteristic in all cases. A few days after transplantation the graft site, considerably removed from the line of skin incision, is swollen and decidedly reddened; the elevation caused by the transplanted tissue, at first scarcely visible, becomes approximately the size of a pigeon's egg or larger. It is typically an inflammatory reaction. Ten days after operation the swelling may be almost as large as three days after transplantation and an active pus discharge may be noted. In many cases, the pus spreads toward and escapes through the healing incision but in many cases erosion of the skin over the site of transplantation may provide an escape for the discharge through the new opening. Pus is often seen exuding from such areas up to the termination of the experiment (30 days). In some cases the transplanted mass has so completely sloughed by the termination of the experiment as to be invisible excepting as the site of transplantation may be marked by scar tissue. In other cases small encapsulated masses of pus are present.

It is evident from these observations and considerations that a

mass of testicular tissues undergoing autolysis after transplantation gives little or no evidence of having liberated hormones into the organism.

V. Discussion.

Our three years experience with the spermatozoön-motility reaction as an indicator for the testis hormone have increased our confidence in the test as a dependable objective test for hormone production. Readily admitting that it lacks several desirable qualities to make it entirely adequate for many different approaches to the subject it must still be recognized as a valuable means of studying hormone production.

Relative to our interests here under discussion, we realize for the first time that the hormone produced by mammal testes is not stored within the body of the organism and the internal secretions of this organ are thus brought into line with such other organs producing internal secretions as parathyroids, pituitary, ovary, etc. In the ovarian follicle it has been realized that a temporary storage, perhaps at the site of production, does occur, but that the body does not ordinarily store it up for future release is shown by the failure of recurrence of œstrous in spayed females. Removal of the testis eliminates the hormone source and there is no evidence that any appreciable quantity is retained within the organism. This is especially emphasized when one realizes that a ten day hormone output by a normal testicle expresses itself with an increase of ten days in the length of sperm life (as indicated by their motility); similar additional periods can in general be detected by the reaction.

When the aspermatogenetic or cryptorchid testicle is studied it is indeed interesting to learn that a testicle reduced in weight to 0.095 grams produces as much hormone as two normal testicles carrying on spermatogenetic activity and weighing approximately 3.4 grams. These cryptorchid testes had originally produced sperm, but due to experimental elevation into the abdomen their germinal epithelium had undergone dissolution and removal. Sections show the typical picture of natural cryptorchid testes in that the seminiferous tubule outlines are reduced to small cavities with the characteristic single-celled layer of Sertoli elements; between the tubules the interstitial

cell masses present the typical picture of "apparent interstitial cell hypertrophy." Such anatomical characteristics have been sufficient stimulus for some writers to designate such an animal as a "supermale" but I have never been able to see adequate justification for the introduction of such a term.

The fact that such cryptorchid testes, having a fraction of the weight of the testicular mass in normal male animals, generated the same quantity of hormone, as do two normal testes (judging from the spermatozoön motility test) suggests again speculation as to the actual elements producing the hormone.

It must be admitted that of all possible elements within the testis, the Leydig cells appear to have the weight of evidence in their favor as being the source of origin of the internal secretions. However, since no one has ever satisfactorily eliminated all other elements such as the general connective tissue, but more especially the cells of Sertoli, there remains the same debatable conditions regarding the actual source of origin. When one views the structure of such degenerate testes as these six and seven month cryptorchid testes (cryptorchid four and five months before epididymal isolation and two months of the experiment) and realizes that each is functioning in producing a full hormone quotient (again judging by the test employed) one is certainly inclined to favor the Leydig cells as the source of origin and to minimize the apparently inactive cells of Sertoli. The question however is not yet settled despite the suggestiveness associated with the Leydig cells.

The chief interest connected with the transplanted testis materials was to see if the spermatozoön motility test would reveal the liberation of any hormone during the process of autolysis of the tissue. Since certain writers had reported such imaginary effects from testis transplantation, it was thought possible that during the breakdown of the incorporated tissues some action might be detected. The results of my investigations, however, have failed to show the liberation of sufficient hormone to be detectable. Despite the capability of the test to reveal hormone action for a period of ten days by a normal testicle it becomes evident that should any hormone effect be derived from transplantation of two entire guinea pig testicles, its effect must be less than that of the normal ten-day production period.

As a further defense of the capability of the spermatozoön motility test to indicate the presence of substances produced by the testicle I may mention that subcutaneous injections of lipoid extracts of the bull testicle, prepared by McGee, and injected by me into guinea pigs whose testes had been removed from their epididymides, resulted in prolonging the life of epididymal sperm to the 54th day after operation (Moore and McGee, '28). Therefore, had the transplanted testis masses been liberating hormone into the host organism, we should have been able to detect it by the test under discussion. Such a result certainly lends no credence to the idea expressed by others that similar non-viable testis grafts in man are sufficiently effective to be noticeable for a period of approximately two years, or again that such transplantations are able to aid in the cure of asthma, tuberculosis, myopia, or the host of other debilities attributed to its effect.

The evidence is very direct that as soon as the hormone producing tissues are removed from the organism the lack of the substance produced begins to be detectable in a very short time. No storage within the body for future utilization is evident.

Summary and Conclusion.

The spermatozoön motility test has been found capable of detecting the production of testis hormone for a period as short as ten days.

The hormone produced by the testicles is not stored within the animal body.

An experimental cryptorchid testicle of five months duration, having a weight of approximately one-tenth of a gram, produces as much hormone (indicated by the spermatozoön-motility test) as do two normal testicles at the height of their spermatogenetic activity. The experimental cryptorchid testis is by weight approximately 2.8 to 3.5 per cent. that of the normal testicular mass.

Autoplastic subcutaneous transplantation of two testes results in the liberation of hormone in such small amounts (if at all) as to be scarcely detectable: if any hormone is liberated by these non-viable testis transplants, the effect upon the animal is no greater than the effect of a ten day normal hormone production. So far as present means will indicate, there is no storage or cumulative effect of the hormone.

BIBLIOGRAPHY.

Benoit, J.

'26 Recherches anatomique, cytologique, et histo-physiologiques sur les voies excretrices du testicule chez les mammiferes. Arch. d'anat. D'physiol. et D'embryol., 5: 173–412.

Bascom, K. F.

'25 Quantitative Studies of the Testis. Some Observations on the Cryptorchid Testes of Sheep and Swine. Anat. Rec., 30: 225–241.

Domm, L. V.

'27 New Experiments on Ovariotomy and the Problem of Sex Inversion in the Fowl. Jour. Exp. Zoöl., 48:

Heller, R. E.

'29 New Evidence for the Function of the Scrotum. Physiological Zoölogy (in press).

Lipschutz, Ottow and Wagner.

'22 On the Hypertrophy of the Interstitial Cells of the Testicle of the Guinea Pig under Different Experimental Conditions. Proc. Roy. Soc., 93: 132–142.

Long, J. A. and Evans, H. E.

'22 The Œstrous Cycle in the Rat and its Associated Phenomena. Memoirs Univ. of California, Vol. 6.

Moore, Carl R.

'21 On the Physiological Properties of the Gonads as Controllers of Somatic and Psychical Characteristics. IV. Gonad Transplantation in the Guinea Pig. Jour. Exp. Zoöl., 33: 365–389.

'22 V. The Effects of Gonadectomy in the Guinea Pig on Growth, Bone Lengths, and Weight of Organs of Internal Secretion. Biol. Bull., 43: 285–312.

'24*a* VI. Testicular Reactions in Experimental Cryptorchidism. Amer. Jour. Anat., 34: 269–316.

'24*b* VIII. Heat Application and Testicular Degeneration, the Function of the Scrotum. Amer. Jour. Anat., 34: 337–358.

'26 IX. Testis-graft Reactions in Different Environments (Rat). Amer. Jour. Anat., 37: 351–416.

'27 A Qualitative Test for the Testis Hormone. Proc. Soc. Exp. Biol. and Med., 24: 847.

'28 X. Spermatozoön Activity and the Testis Hormone. Jour. Exp. Zoöl., 50: 455–494.

Moore, Carl R., and Lemuel C. McGee.

'28 On the Effects of Injecting Lipoid Extracts of Bull Testes into Castrated Guinea Pigs. Amer. Jour. Physiol., 87 (in press).

Moore, Carl R., and Wm. J. Quick.

'24 The Scrotum as a Temperature Regulator for the Testes. Amer. Jour. Physiol., 78: 70–79.

Stockard, C. R., and G. N. Papanicolau.

'17 The Existence of a Typical Œstrous Cycle in the Guinea Pig with a Study of its Histological and Physiological Changes. Amer. Jour. Anat., 22: 225–264.

Stone, Calvin P.

'27 The Retention of Copulatory Ability in Male Rats following Castration. Jour. Comp. Psychology, 7: 369–387.

INITIATION OF DEVELOPMENT IN *ARBACIA*. V

The Effect of Slowly Evaporating Sea-Water and its Significance for the Theory of Auto-Parthenogenesis.*

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In 1901 Hunter published results of experiments which showed that uninseminated eggs of *Arbacia* exposed to sea-water concentrated by evaporation develop on return to normal sea-water. The present writer has been able to confirm these results though his method differs from Hunter's. The sole reason for reporting the findings here presented is that they lead to some interpretations of significance for Lillie's fertilizin theory of fertilization. The work was done during several summers spent at the Marine Biological Laboratory, Woods Hole, Mass.

The Experiments.

Normal uninseminated eggs of *Arbacia*—free of perivisceral fluid, of high fertilizin content, and capable of giving one hundred per cent. fertilization and cleavage—show a small per cent. of cleavage and of abnormal blastulæ that do not rise to the surface, if after having lain in a small volume of normal sea-water for one or more hours, they are removed to a larger volume of normal sea-water. Two conditions are important for this method of initiating development in the egg of *Arbacia*. First, it is best to use fairly dense egg suspensions. The less dense suspensions prolong the time of exposure necessary to initiate development. Secondly, *it is indispensable for the experiment that the dish containing the eggs be left uncovered to insure evaporation.* A concentration of 1 cc. of "dry" eggs plus 99 cc. of normal sea-water was the least dense suspension successfully used. In some cases it was necessary to leave such a suspension uncovered for twenty-four hours before transfer to the larger volume of sea-water; but it was clear here that evaporation was

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responsible since suspensions of this kind always gave better results when placed in larger dishes with greater surface for evaporation. And if, moreover, a 1 cc. suspension of eggs in 100 cc. of sea-water be poured on a glass plate thereby insuring greater evaporation, the results were indeed striking. However, I am here interested mainly in the results obtained with smaller volumes of eggs and of sea-water.

In all the experiments it was first ascertained that the eggs to be used were in optimum fertilizable condition by trial inseminations for the estimation of their capacity to separate normal membranes. First, the eggs were carefully collected uncontaminated by perivisceral fluid, washed in four changes of 200 cc. of sea-water, and allowed to settle. The supernatant sea-water was decanted and a highly concentrated bulk of eggs thus obtained. For each experiment the eggs were from one female. These were divided into two equal lots whenever their bulk permitted; one lot was placed in an open dish, the other in a glass vial closed with a ground glass stopper. At intervals of 30 minutes a drop of eggs from each lot was removed to 250 cc. of normal sea-water and their development observed. The per cent. of cleavage was as carefully counted as possible, though the count is often made difficult because of the number of cytolyzed eggs. With further development complete cytolysis of eggs makes the counting of "swimmers" more difficult and of doubtful value since these counts cannot take into consideration eggs that have disappeared through complete disintegration. Moreover, many of the eggs exposed to evaporation develop with their blastomeres separated because the eggs do not possess membranes. In such cases, two "micro-blastulæ" counted may have developed from one egg or each from a different egg, its fellow mass of blastomeres having disappeared completely through disintegration. I therefore early abandoned attempts at making accurate counts and merely noted the presence or absence of "swimmers."

I wish to emphasize that in not one single experiment did I ever find an egg in the stoppered vial that showed the least sign of development. That these eggs were not impaired I determined by inseminating them—in normal sea-water in the case

of highly concentrated suspensions, or in the vials in the case of the less concentrated suspensions.

One other point before we consider the experiments in detail. The reader appreciates the fact that the rate of evaporation varied from day to day. I made no attempt to control this variation. It is also obvious that the rate of evaporation depends upon the volume of solution employed—smaller volumes evaporating more rapidly than larger. Finally, the vessels used make a difference; in my experiments I used either shallow dishes, with a large surface for evaporation, or for the greatest volumes of solutions employed glass plates, 30 x 30 cm. For volumes up to 4 cc. Syracuse watch glasses served admirably.

There now follow a summary (Table I.) of the first type of experiment and a brief comment for the purpose of elucidation.

TABLE I.

THE EFFECT OF SLOWLY EVAPORATING SEA-WATER ON THE UNINSEMINATED EGGS OF *Arbacia* AS SHOWN BY THE PER CENT. OF THEIR DEVELOPMENT ON RETURN TO NORMAL SEA-WATER. EXPERIMENTS ON EGGS OF 45 FEMALES

No.	Bulk of Concentrated Eggs (in cc.).	Volume of Sea-water (in cc.).	Per Cent. of Cleavage.						Per Cent. of "Swimmers."
			Exp. No.	1	2	3	4	5	Exp. No. 1.
1	0.1	0.9		8	11	7	14	20	9
2	0.5	0.5		7	0	11	14	3	5
3	0.6	1.2		6	12	4	9	0	4
4	1	1		0	6	9	7	2	0
5	1	1		10	7	8	4	14	13
6	1.5	3		21	17	23	27	18	23
7	2	2		0	3	4	0	5	0
8	2.5	5		13	6	18	21	19	15
9	3	3		14	24	5	0	6	10

Eggs from the same females in stoppered vials: No cleavage, no "swimmers" Same volumes of eggs and sea water in each case except as follows: No. 7, 0.5 cc. of eggs + 0.5 cc. sea-water; No. 8, 0.5 cc. of eggs + 0.5 cc. of sea-water; No. 9, 0.5 cc. of eggs + 1 cc. of sea-water.

The data given in Table I. are for eggs exposed to slowly evaporating sea-water for two hours. This one length of exposure is arbitrarily taken for the purpose of simplicity, instead of presenting the results of each 30-minute exposure. In some

instances the per cent. of development was greater after a longer or a shorter exposure; the results of the two-hour exposure is very nearly the average of all exposures made. Though the per cent. of development in no experiment is high, yet it shows that the evaporating sea-water does initiate development. I do not regard this as an efficient method for experimental parthenogenesis: it has been very suggestive, however, for other lines of my work.

Eggs in sea-water protected against evaporation never show indication that development is initiated. This statement is certainly superfluous for suspensions of uninseminated eggs of *Arbacia* that are ordinarily employed as controls, as all workers know. Of the more dense egg suspensions it might be that lack of oxygen or CO_2 concentration makes initiation of development impossible. The fact that such eggs from such suspension fertilize on return to larger volumes of sea-water does not meet this possible objection. However, I might repeat that some suspensions made of 1 cc. of eggs plus 99 cc. of sea-water exposed to slowly evaporating sea-water showed initiation of development whereas similar suspensions in stoppered vials never did.

Eggs that show initiation of development as the result of exposure to evaporating sea-water never separate membranes, their cleavage is irregular, and the blastomeres tend to fall apart. Many eggs do not cleave and of these some reach the monaster stage with rhythmical dissolution and re-formation of the nucleus. All uncleaved eggs on insemination separate membranes, cleave, and reach the pluteus stage.

The abnormal swimming forms developing from these eggs subjected to treatment with slowly evaporating sea-water never swim at the surface, but merely rotate on the bottom of the dishes; among them are micro- and mega-"blastulæ"—*i.e.*, swimming forms developed from blastomeres that have fallen apart and those developed from two or more cleaving eggs. It is this fact that makes difficult the counting of swimmers; hence, the reader will note that except for the first experiment (Table I.) and for one experiment described below (Table II.), I give no counts, but simply note with a + or o sign their presence or absence.

I interpret these experiments to mean that these eggs in

evaporating sea-water are by such evaporation exposed to hypertonic sea-water. It is the hypertonicity that is responsible for the initiation of development and not the mere crowding of the eggs since equivalent volumes of eggs from the same females and of sea-water protected against evaporation do not give any evidence of initiation of development after transfer to larger volumes of sea-water. These eggs as noted above had been thoroughly washed before exposure to evaporation; they would nevertheless continue to produce fertilizin—but so would the eggs protected against evaporation. If fertilizin production, therefore, were responsible for the initiation of development we might expect that at least the highly concentrated eggs in stoppered vials would show some signs of cleavage and farther development. And, what is more, the use of "egg water" instead of normal sea-water does not increase the per cent. or improve the development. Table II. gives the results of a typical experiment on eggs exposed to evaporating "egg water." Drops of eggs from both the uncovered and the stoppered lots were returned at half hour intervals to 200 cc. of normal sea-water. The percentages given are those of eggs having had a two-hour exposure to the evaporating "egg water." This experiment was made five times.

TABLE II.

THE EFFECT OF SLOWLY EVAPORATING EGG WATER ON THE UNINSEMINATED EGGS OF *Arbacia* AS SHOWN BY THE PER CENT. OF THEIR DEVELOPMENT ON RETURN TO NORMAL SEA-WATER: EXPERIMENTS ON THE EGGS OF 9 FEMALES.

No.	Bulk of Concentrated Eggs (in cc.).	Volume of Egg Water (in cc.).	Per Cent. of Cleavage.	Per Cent. of "Swimmers."
1	0.5	1.5	9	7
2	0.5	2.5	13	11
3	1	1	7	0
4	1	1	18	14
5	1	2	11	5
6	2	1	3	5
7	2	2	15	12
8	2	3	12	10
9	2.5	5	9	6

Equivalent volumes of eggs from the same females and of "egg-water," except for No. 9 where 0.5 cc. of eggs and 1 cc. of "egg water" were used, in stoppered vials gave no trace of development after return to normal sea-water.

It would appear from a study of Table II. that there is no advantage in substituting "egg water" for sea-water. As a matter of fact, other experiments with "egg water" gave inferior results. In addition, one gains the impression that exposure to evaporating "egg water" causes more eggs to separate blastomeres, and that there are more micro- and mega-"blastulæ." This I did not properly investigate, *i.e.*, by running experiments on lots of concentrated eggs from the same females, one lot exposed to evaporating sea-water, one to stoppered sea-water, one to evaporating "egg water," and one to stoppered "egg water" counting both the eggs that showed blastomeres falling apart and the micro-"blastulæ." However, some older unpublished observations made independently by Lillie and by the writer may be cited. These showed that "egg water" actually possesses a deleterious effect on development. Thus, I found that if eggs from the same female be divided into two lots, one suspended in sea-water and the other in strong "egg water" before or after insemination, the development of the eggs in "egg water" are markedly inferior to that of the eggs in normal sea-water as measured by the per cent. and normality both of cleavage and of plutei. Lillie also has commented on the adverse effect of "egg water" in other ways on eggs. There is indeed no reason why this should not be true and several reasons why it should. "Egg water" is not simply sea-water charged with fertilizin—it contains products of metabolism of the uninseminated eggs, even though metabolism is at a low level; this would be especially true of eggs highly concentrated in strong "egg water," which perhaps also contains more bacteria than normal sea-water.

Glaser likewise notes that "addition of the extracts ["egg water"] in certain concentrations to normally fertilized eggs, resulted in a retardation of development; normal blastulæ instantly slowed their movements, and underwent a noticeable increase in volume when subjected to the extracts. Similar observations were made on the larvæ of *Arenicola* whose rate of movement was also slowed down, to be followed instantly by an outflow of their yellow pigment and a slight reversible agglutination." Unfortunately, however, Glaser's method of preparing his egg extracts—by removing the eggs directly from the ovaries

into twice their volume of sea-water—is open to objection since he must have carried over some perivisceral fluid. The perivisceral fluid alone may have been responsible for his results.

The following experiment was also made ten times: eggs from one female were placed (1) in sea-water exposed to evaporation, (2) in sea-water in a stoppered vial, (3) in "egg water" exposed to evaporation and (4) in "egg water" in a stoppered vial; at 30 minute intervals drops of eggs were removed from each of the four lots to dishes each containing 200 cc. of normal sea-water. I give now the summary of one long experiment because it shows the results with varying concentration of eggs from one female:

No. 1. 10 drops of eggs + 90 drops of uncovered sea-water gave 18 per cent. cleavage, + + "swimmers."

No. 2. 10 drops of eggs + 90 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 3. 10 drops of eggs + 90 drops of uncovered egg water gave 6 per cent. cleavage, + "swimmers."

No. 4. 10 drops of eggs + 90 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 5. 20 drops of eggs + 80 drops of uncovered sea-water gave 27 per cent. cleavage, + + "swimmers."

No. 6. 20 drops of eggs + 80 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 7. 20 drops of eggs + 80 drops of uncovered egg-water gave 8 per cent. cleavage, + "swimmers."

No. 8. 20 drops of eggs + 80 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 9. 30 drops of eggs + 70 drops of uncovered sea-water gave 31 per cent. cleavage, + + "swimmers."

No. 10. 30 drops of eggs + 70 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 11. 30 drops of eggs + 70 drops of uncovered egg water gave 11 per cent. cleavage, + "swimmers."

No. 12. 30 drops of eggs + 70 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 13. 40 drops of eggs + 60 drops of uncovered sea-water gave 21 per cent. cleavage, + + "swimmers."

No. 14. 40 drops of eggs + 60 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 15. 40 drops of eggs + 60 drops of uncovered egg water gave 17 per cent. cleavage, 0 "swimmers."

No. 16. 40 drops of eggs + 60 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

In this experiment because of the rapidity of evaporation on this particular day the eggs were removed to normal sea-water after one hour. The experiment reveals that the effect of evaporating "egg water" is certainly not superior to that of evaporating sea-water in causing initiation of development. It shows also as other experiments cited show that more concentrated suspensions do not yield markedly higher percentages of development than less concentrated ones.

On the whole I think that the evidence which I have submitted indicates that eggs exposed in uncovered dishes develop because of an increasing hypertonicity due to evaporation. Further, the evidence indicates that "egg water" is not necessary for this effect; indeed, "egg water" appears to be less efficacious if not actually more harmful in some small degree than normal sea-water. If this evidence be accepted, Glaser's work on auto-parthenogenesis must be questioned. A brief discussion of Glaser's work and its significance for the fertilizin theory in the light of the work which I herein report now follows.

Discussion.

In 1914 Glaser reported for eggs of *Arbacia* and *Asterias* a type of initiation of development due to exposure to "egg water" for which he chose the name, auto-parthenogenesis. Glaser's procedure was as follows: "Standard secretion ["egg water"] was prepared by adding to a certain number of "dry" ripe ovarian eggs, double their volume of sea-water. At the end of ten minutes, during which the eggs were slightly agitated at intervals, the suspension was centrifuged, and the eggs cast down. After 100 revolutions the supernatant fluid was carefully decanted and set aside for use.

"Ripe eggs were then shaken, usually from the ovaries of a single individual, into a small quantity of fresh sea-water, and to 1 cc. of a concentrated suspension of these was added 1 cc. of the secretion. In this mixture the eggs were allowed to stand 2 hours, when cleavages were usually found in all the dishes."

And further: "Many experiments were tried varying the concentration of the secretion as well as the time of exposure. My records indicate cleavages at higher concentrations as well as lower, and also in less than two hours, but the greatest number

was always obtained when 1 volume of the concentrated egg suspension was exposed for 2 hours to 1 volume of the standard secretion. If at the end of this time the supernatant fluid is poured off and replaced by fresh sea-water, free swimming blastulæ will be found within 24 hours. In one case only did development proceed to the pluteus stage."

As I have stated above, Lillie was never able to repeat this observation made by Glaser. Nor was I until by chance I observed the extent of evaporation that had taken place in two cc. of egg water put in a Syracuse watch glass one hour before. Deliberately repeating this chance observation on eggs suspended in either "egg water" or sea-water through several seasons I have obtained initiation of development in *Arbacia* eggs provided the "egg water" or sea-water be allowed to evaporate. I am therefore constrained to believe that Glaser's auto-parthenogenesis is a hypertonic effect due to evaporation.

Glaser has also reported what he calls an improved method of auto-parthenogenesis. Says Glaser: "Loeb's improved method of artificial parthenogenesis consists in following the treatment with parthenogenetic agents, by an after treatment with hypertonic sea-water, 8 cc. of 2.5 *M* NaCl plus 50 cc. of sea-water. It seemed likely, therefore, that a better yield of larvæ could be secured if eggs, after having been subjected to the action of the secretion for two hours, were afterwards treated with the hypertonic solution for forty minutes. This surmise proved correct." The proof offered is the outline of a typical experiment showing the development in two sets of eggs *both* of which were exposed to hypertonic sea-water after treatment with the egg secretion. There are, it seems to me, two objections to this experiment.

In the first place, in the improved method of artificial parthenogenesis Loeb typically used butyric acid which alone is not capable of causing development of the egg beyond the separation of the vitelline membrane and formation of a monaster around the egg nucleus; according to Glaser, the egg secretion which he used causes development at least to the blastula stage without separation of membranes. Moreover, when one uses butyric acid one must replace the acid sea-water with normal sea-water and allow a certain time to elapse before beginning the treatment

with the hypertonic sea-water; Glaser exposed his eggs to the egg secretion and at once transferred them to the hypertonic sea-water. There is here, therefore, no similarity between the improved method of Loeb and that of Glaser.

Secondly, and this is far more serious, Glaser does not tell us to what extent there is an improvement through the after treatment with the hypertonic sea-water; he gives no information concerning the development of two lots of eggs from the same female, one with and one without hypertonic sea-water after the exposure to the egg sea-water. Obviously, Glaser should have set up an experiment on four lots of eggs from the same female, one an uninseminated control in normal sea-water, one exposed to hypertonic sea-water alone, one to egg water alone, and one to hypertonic solution after a treatment with "egg water."

In the same communication Glaser also described autoparthenogenesis in eggs of *Asterias*. For this he used either 1 or 2 volumes of maturing *Asterias* eggs plus one of "egg water" and obtained fertilization membranes, cleavage, and "much gastrulation." I would suggest that this result was due in part to CO_2, which in *Asterias* eggs initiates development, and to hypertonicity.

Glaser's "hetero-parthenogenesis" is the effect of *Arbacia* "egg water" on *Asterias* eggs. Here again he used 1 volume of "egg water" (from *Arbacia* eggs) to 1 volume of *Asterias* eggs. The foreign "egg water" gave fertilization membranes and numerous cleavages. I venture the opinion that the initiation of development was due to one, two or a combination of three factors: CO_2, hypertonicity, and the foreign perivisceral fluid which from Glaser's method of procuring the *Arbacia* "egg water" must have been present.

On the basis of my findings and the possibility that these adverse criticisms of Glaser's work be correct, I suggest that autoparthenogenesis is an initiation of development due to hypertonicity of either "egg water" or sea-water. If this be true Glaser's criticisms of Lillie's fertilizin theory based on his findings are without foundation.

LITERATURE CITED.

Glaser, Otto.

'14 On Auto-parthenogenesis in *Arbacia* and *Asterias*. BIOL. BULL., 26, pp. 387–409.

Hunter, S. J.

'01 On the Production of Artificial Parthenogenesis in *Arbacia* by the Use of Sea-water Concentrated by Evaporation. Amer. Jour. Physiol., 6, pp. 177–180.

Lillie, Frank R.

'14 Studies of Fertilization. VI. The Mechanism of Fertilization in *Arbacia*. Jour. Exp. Zoöl., 16, pp. 523–590.

INTRACELLULAR HYDRION CONCENTRATION STUDIES.

I. The Relation of the Environment to the pH of Protoplasm and of its Inclusion Bodies.

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Recent micrurgical investigations (1, 2, 3) on the colorimetric determination of the protoplasmic pH have emphasized the need of studying the relation between the pH of the protoplasm of a living cell and that of its environment. Of the acids and bases which affect the pH of the environment some penetrate living cells while others apparently do not. This has been demonstrated by the change in color of cells stained with indicators. For example, with the use of neutral red it has been shown by previous investigators (4, 5) that living cells are readily permeable to CO_2 and NH_3 but not to HCl nor NaOH. This fact that the color of the intracellular stain can be readily shifted to the acid or the alkaline side suggests that the intraprotoplasmic pH can be changed very easily by environmental conditions, a conclusion which is at variance with experiments which indicate that protoplasm has a marked buffering power. Thus, when solutions of indicators, both in the acid and the alkaline states of their color ranges, are injected into living cells the colors quickly shift to those characteristic of a constant pH (6.9 ± 0.1). This has been found true for such varied types of cells as the ameba (1, 6), marine ova (2, 3), and various tissue cells of the frog and the mammal (6). In addition, there is the significant result that the localized increase in intraprotoplasmic acidity, caused by mechanical injury is almost immediately neutralized as long as no cytolysis results (1, 2, 3, 6).

In view of these facts it was considered advisable to test further the constancy of the intraprotoplasmic pH, to discover

whether this pH can be shifted appreciably without detriment to the cell and to obtain evidence, if any, of localized variations in the intracellular pH.

The purpose of the experiments described in this paper is to determine whether the intraprotoplasmic pH can be shifted by exposure to CO_2 or to NH_3 and whether the reaction to indicators of such intracellular structures as granules and vacuoles are comparable to those of the optically homogeneous protoplasmic matrix.

Before dealing with the actual experiments performed it is necessary to describe the manner in which the protoplasm becomes colored with neutral red and with the other dyes used. When cells are stained with neutral red or certain other basic dyes, the dye accumulates in or on the intracellular granules and vacuoles while the hyaline protoplasmic matrix remains colorless. This occurs not only when cells are stained by immersion in a solution of the dye but also when the dye is injected directly into the cell. In the latter case the color appears at first diffuse but gradually the granules and vacuoles take up more and more of the color until none of it can be detected in the hyaline cytoplasmic matrix. On the other hand the acid dyes used, *e.g.*, brom cresol purple, phenol red and cresol red, do not penetrate from the environment into the cells. When injected, however, they quickly spread through the cytoplasm giving to its hyaline matrix a more or less permanent and diffuse coloration (1, 2, 3, 6).

The fresh water *Amœba dubia* and the unfertilized eggs of the starfish, *Asterias forbesii*, and sanddollar, *Echinarachnius parma*, were used in these experiments. The amœba and the eggs were colored with the dyes either by the immersion method or by the microinjection method. Both methods were also used simultaneously on the same cell. The cells were then immersed in various acid and alkaline solutions and the color changes noted. For a study of the effect of NH_3 and CO_2 the cells were suspended in hanging drops of water from the roof of a special form of moist chamber which was closed except for narrow inlet and outlet tubes (7). The hanging drops were then charged with either CO_2 or with NH_3 by passing the moist gas through the chamber.

1. Effect of Acids and Bases on Amebæ Colored by the Injection of Acid Indicators Only.

Amebæ were injected with 0.4 per cent solution of brom cresol purple, phenol red and cresol red (8). These indicators were selected because they change color within the pH ranges tested (1, 3). Amebæ, injected with brom cresol purple, are uniformly blue (the alkaline range), with phenol red, a pale orange yellow (approaching the acid range). These findings accord with those already published (6) from which the pH of the freshwater ameba was placed at 6.9 ± 0.1.

Amebæ, colored by the injection of the above-mentioned dyes, were immersed in solutions of HCl (pH 5.5), NH_4Cl (pH 5.5), CO_2 charged water (pH 5.5), $NaHCO_3$ (pH 8), NH_4OH (pH 8) and NaOH (pH 8). The acidity of the first three solutions is sufficient to cause the indicators to take on the yellow color of their acid ranges, while the alkalinity of the last three solutions is sufficient to give to brom cresol purple the purple blue, and to phenol red and cresol red the bright red color of their alkaline ranges. It was found that the immersed amebæ all maintained their original colors as long as they remained alive. The color of those which rounded up and died changed to that characteristic for the pH of the environing medium.

These results indicate, either that there is no penetration of the acid or of the alkali from the solutions used, or that the protoplasm is sufficiently buffered to neutralize the acid or the alkali which does penetrate.

2. Effect of Acids and Bases on Cells Stained with Neutral Red and Injected with Acid Indicators.

a. Amœba dubia.

Since the permeability of cells to certain acids and bases can be demonstrated by the change in color of neutral red, amebæ were immersed in a solution of neutral red until various intracellular inclusions took on a red color. These amebæ were then injected with solutions of the indicators which color the cytoplasm diffusely. On immersing these doubly colored amebæ into the various acid and alkaline solutions the following results were obtained:

In accordance with the previous experiment it was found that immersion produced no change whatever in the diffuse coloration of the hyaline cytoplasmic matrix. On the other hand, the inclusion bodies which were stained with neutral red quickly became yellow in the solutions containing the NH_3 (NH_4OH and NH_4Cl) and bright red in those containing CO_2 ($NaHCO_3$ and CO_2 charged water).

These results imply that the pH of the hyaline cytoplasm does not change even when sufficient NH_3 or CO_2 penetrates to change the color of the intracellular inclusions. In other words, the pH of the intracellular inclusions can be shifted readily by the presence of CO_2 or of NH_3 in the environment while that of the protoplasmic matrix remains constant.

b. Unfertilized Eggs of the Sanddollar (Echinarachnius parma) and the Starfish (Asterias forbesii).

The protoplasm of these eggs is uniformly crowded with granules or macrosomes practically all of which ultimately stain a deep rose red with neutral red. The eggs were allowed to remain in sea-water containing neutral red only long enough to stain a small percentage of the granules. The eggs were then washed, transferred to hanging drops of sea-water in the moist chamber and injected with the indicator solutions. In the same chamber were placed, as controls, other hanging drops of sea-water colored with the same indicators. Ammonia gas was then passed through the chamber until the hanging drops became sufficiently saturated with ammonia to change the color of the control drops.

The color of the eggs was noted when the dyes in the control drops had assumed colors indicating a pH more alkaline than 8.4. In every case the color of the granules, stained with neutral red, changed from red (acid) to yellow (alkaline) while the diffuse coloration of the indicators in the hyaloplasm of the eggs persisted in registering the originally recorded pH of 6.8 ± 0.1 (3).

An experiment giving striking color contrasts is one in which three dyes, neutral red, phenol red and cresol red, were used for the purpose of detecting simultaneously the pH changes in the cytoplasm, the cytoplasmic granules, and the sea-water surrounding the eggs. It is to be remembered that neutral red

which stains the granules is red at a pH more acid than 6.8 and yellow at a pH more alkaline than 7.4. Phenol red which colors the hyaloplasm is yellow at a pH more acid than 6.8 and red at a pH more alkaline than 7.4, and cresol red which was used for the environing sea-water is yellow at a pH more acid than 7.8 and red at a pH more alkaline than 8.0. The experiment was the following: Eggs, stained with neutral red, were immersed in a hanging drop of sea-water colored with cresol red and were then injected with phenol red. The result was a striking picture of yellow eggs containing scattered red granules and surrounded by a medium of yellow sea-water. Ammonia gas was then passed through the chamber until the cresol red in the sea-water changed from yellow (acid) to red (alkaline). As soon as this occurred the cytoplasmic granules, stained with the neutral red turned yellow (alkaline) while the hyaloplasm maintained the original yellow (acid) color of the phenol red. The result was now a picture of uniformly yellow eggs standing out against a background of red sea-water. Carbonic acid gas was then passed through the chamber until it displaced the NH_3 in the hanging drops. As a result the original colors returned, viz., the sea-water again became yellow, the cytoplasmic granules turned from yellow to red but the cytoplasm itself remained yellow.

Since the cytoplasm has a pH of 6.8 ± 0.1 (3) which is in the acid range of phenol red the above experiment is not suited for detecting a possible effect of the CO_2 on the cytoplasmic pH. For this purpose it is necessary to use brom cresol purple (yellow at a pH more acid than 6.0 and blue at a pH more alkaline than 6.2) which, upon injection, colors the hyaloplasm blue. These eggs were immersed in a hanging drop of sea-water colored blue with the same dye. The hanging drop was suspended in the hermetic chamber through which moist CO_2 gas was made to stream until the sea-water became charged with CO_2 sufficiently to change its color from blue to yellow. The eggs in the yellow water kept their original blue color.

These experiments indicate that NH_3 and CO_2, both of which penetrate the protoplasm and affect the pH of the intracellular granules, do not shift the pH of the hyaloplasm as measured by the indicators.

3. Effect of CO_2 and of NH_3 on Amebæ whose Cytoplasm and Inclusion Bodies are Colored with the Same Indicator.

A possible error in the previous experiments lies in the fact that the coloration of the cytoplasmic inclusions and of the hyaline cytoplasm were not made with the same dye. For example, neutral red, which colors the cellular inclusions, is a basic dye, while the dyes used for producing a diffuse coloration are acidic. It is conceivable that this may be responsible for their difference in reaction to the penetrating CO_2 or NH_3.

To meet this objection it was found that methyl red could be used. Methyl red has already been used as a vital stain for plant protoplasm (9) and is a pH indicator, being red at a pH more acid than 5.0 and yellow at a pH more alkaline than 5.4. Immersion of amebæ in an aqueous solution of this dye stains the hyaline cytoplasm, its various inclusions and the nucleus an intense yellow. Amebæ colored in this way were placed in a moist chamber in hanging drops of the yellow aqueous solution of methyl red. Moist CO_2 gas was then passed through the chamber until the hanging drops turned from yellow to red. When this occurred it was found that the yellow stained inclusions of the ameba had also become red while the cytoplasm and nucleus remained yellow. Ammonia vapor was now passed through the chamber whereupon the color of the hanging drops and of the intracellular inclusions quickly changed back to yellow.

These experiments with methyl red clearly demonstrate the penetration of CO_2 into the living ameba [1] as registered by the change in color of the intracellular inclusions. The hyaline cytoplasm and the nucleus, however, maintain their original color and give no evidence of a change in pH.

[1] The neutral red method is not very favorable for detecting the penetration of CO_2 into cells since the granules stained with neutral red under normal conditions already have the rose red color characteristic for the acid range of the dye. On the other hand, methyl red under normal conditions stains the intracellular granules the yellow color of its alkaline range. Upon exposure to CO_2 the color of the granules changes to red, which is as decided an evidence for the penetration of the CO_2 as is the neutral red method for the penetration of NH_3.

4. The Effect of Penetrating Acids and Bases on the Nuclear pH.

The nuclei of immature starfish eggs were used in these experiments. The nuclei of different eggs were colored with cresol red, neutral red and phenol red by the microinjection method after which the eggs were exposed to CO_2 and to NH_3. In every case the color within the nuclei of living eggs remained constant irrespective of the color changes of the granules in the surrounding cytoplasm. In other words, the nucleus was found to be sufficiently buffered so that the intranuclear pH of 7.6–7.8 (3) remains unchanged. When the egg disintegrates by crushing or tearing, the nucleus undergoes changes (3) and loses all buffering action. The persisting spherical nuclear remnant is then immediately susceptible to acid and alkali changes in its environment.

Summary.

The presence of CO_2 or of NH_3 in the aqueous medium surrounding living cells readily changes the pH of the intracellular inclusions which stain with neutral red but does not change the pH of the protoplasmic matrix nor of the nucleus as long as the cell is alive.

Bibliography.

1. **Needham, J., and Needham, D. M.**
'25 The Hydrogen-ion Concentration and the Oxidation-reduction Potential of the Cell-interior: A Micro-injection Study. Proc. Roy. Soc., B, 98, 259.

2. **Needham, J., and Needham, D. M.**
'26 The Hydrogen-ion Concentration and Oxidation-reduction Potential of the Cell-interior before and after Cleavage: A Micro-injection Study on Marine Eggs. Proc. Roy. Soc., B, 99, 173–199.

3. **Chambers, R., and Pollack, H.**
'27 Micrurgical Studies in Cell Physiology. IV. Colorimetric Determination of the Nuclear and Cytoplasmic pH in the Starfish Egg. Jour. Gen. Physiol., 10, 739–755.

4. **Harvey, E. N.**
'14 The Relation between the Rate of Penetration of Marine Tissues by Alkali and the Change in Functional Activity Induced by the Alkali. Publ. Carneg. Instit., Wash., No. 183, 131.

5. **Jacobs, M. H.**
'20 The Production of Intracellular Acidity by Neutral and Alkaline Solutions Containing Carbon Dioxide. Amer. J. Physiol., 53, 457.
'22 The Influence of Ammonium Salts on Cell Reaction. J. Gen. Physiol., 5, 181.

6. **Chambers, R., Pollack, H., and Hiller, S.**
'27 The Protoplasmic pH of Living Cells. Proc. Soc. Exp. Biol. and Med., 24, 760–761.
7. **Cohen, B., Chambers, R., and Reznikoff, P.**
'28 Intracellular Oxidation-reduction Studies, I. J. Gen. Physiol, II, 585.
8. **Clark, W. M.**
'25 The Determination of Hydrogen Ions. Williams and Wilkins Co., Baltimore, 2d ed.
9. **Schaede, R.**
'24 Über die Reaktion des lebenden plasmas. Ber. d. bot. Ges., 42, 219.

INTRACELLULAR HYDRION CONCENTRATION STUDIES.

II. The Effect of Injection of Acids and Salts on the Cytoplasmic pH of *Amœba dubia*.[1]

PAUL REZNIKOFF and HERBERT POLLACK.[2]

In a previous communication (1) from this laboratory the pH of the cytoplasm of *Amœba dubia* was reported to have a value of 6.9 ± 0.1. To determine whether any permanent variations in the intracellular pH could be artificially produced, solutions of acids salts and simple salts having toxic actions were injected by the micrurgical technique (2) into amebæ previously colored with indicators.

Experimental.

The ameba and methods used in these experiments were the same as those described previously (2). The hydrion indicators (3) employed were thymol blue, brom phenol blue, brom cresol green, methyl red, chlor phenol red, brom cresol purple, phenol red, and orange III. Of these phenol red was the most extensively used. The advantages of this dye are twofold. It is the least toxic of all the indicators and is the most valuable one in experiments of this type since its useful range covers the normal cytoplasmic pH. The other indicators were used in extreme changes of pH.

In the case of each solution, at least 10 amebæ were used, and for critical concentrations from 25 to 50. Small quartz cover slips were employed in these experiments. They were attached by means of water films to the ordinary long glass cover slips. On the quartz slips were placed amebæ in a drop of their medium, varying in reaction from pH 5.8 to 7.5, a drop of indicator, and

[1] From the Laboratory of Cellular Biology, Department of Anatomy, Cornell University Medical College, New York City, and the Marine Biological Laboratory, Woods Hole, Massachusetts.

[2] Expenses connected with this investigation were in part defrayed from a grant by the Ella Sachs Plotz Foundation.

a drop of the solution the effect of which was to be tested. The pipettes used were made of pyrex glass and were rinsed several times in distilled water and then in solutions of the substances to be injected. The dyes were injected into the amebæ which were permitted to recover. After recovery the next solutions were introduced.

As a control, the degree of injury caused by the insertion of the pipette was determined. The method employed was to note any change in color of the previously injected dye from the possible formation of acid associated with injury (4). The simple introduction of a pipette into an ameba was found to give no indication of acid production. If the pipette, however, stirred up the cytoplasm so vigorously that the injured area was ultimately discarded, a distinct acidity was produced. When death occurred in the presence of those dyes covering the range, a pH of about 5.5 was indicated, unless the pellicle surrounding the mass was broken in which case the color was rapidly washed out.

Solutions of HCl (pH 2) when introduced into an ameba, which is colored an orange-yellow with previously injected phenol red, cause an immediate and intense yellow coloration of the injected area. If the injected region is not irreparably injured by the acid the pH of the area reverts within a few seconds to that of the normal cytoplasm. Usually, however, the injected portion is injured to such an extent that it is pinched off in a manner previously described (2) after which the yellow color (acid) of the discarded sphere gradually changes to that indicative of the pH of the environment. In time the color entirely washes out. The unaffected remnant of the ameba retains its orange-yellow color.

When $CaCl_2$ is injected in concentrations stronger than $M/200$ the phenomenon of solidification and pinching off is accompanied by distinct evidence of acid production. If a solution of $M/200$ $CaCl_2$ is injected into amebæ colored with phenol red, the flash of yellow color indicating acid production rapidly returns to that of the normal pH, provided the injected area is not discarded. If the color does not revert within a few seconds the affected portion is pinched off.

In only three cases out of several hundred did the maintenance of a localized acid reaction persist for as long as a minute after HCl or $CaCl_2$ had been introduced without subsequent pinching off. To investigate further this condition in which a localized acid reaction is maintained for an appreciable time with subsequent complete recovery solutions of $M/32$ $AlCl_3$ were introduced into amebæ colored with phenol red. Such a concentration of $AlCl_3$ causes a solidification of the injected portion but this region is not infrequently reincorporated after being almost discarded (5). Of at least 50 cases only one showed a delay of two to three minutes in the return of color from yellow (acid) to the original orange-yellow after the affected area had been reincorporated. In every other test the reversal of color was immediate if reincorporation occurred or, if the area was discarded, its color remained yellow.

The introduction of solutions of $MgCl_2$ of pH 6.5 in concentrations of $M/30$ and stronger into amebæ previously injected with phenol red causes an immediate shift to yellow, indicating acid production. The cell breaks and the color diffuses out. When an $M/60$ solution of $MgCl_2$ is injected the yellow color reverts rapidly to that normal for healthy cytoplasm and the ameba recovers.

In order to determine the degree of acid production by the injection of $CaCl_2$ and MgCl (pH 6.6) amebæ were injected with this salt after having been colored with thymol blue, orange III, methyl red, brom phenol blue and brom cresol green. All these dyes were injected with the exception of methyl red (6). Amebæ were stained with methyl red by immersing them into 5 cc. of distilled water into which a few drops of a 0.4 per cent. aqueous solution of methyl red were placed. With methyl red a distinct red is produced when either $CaCl_2$ or $MgCl_2$ is injected into amebæ. With orange III the yellow color persists. This places the reaction of the acidified portion of the cell between pH 4.0 and 4.6. It is difficult to determine a more exact pH value because the color changes with brom phenol blue are not sufficiently distinctive within the critical range. These results show that the acid produced by injection of $CaCl_2$ or $MgCl_2$ is more marked than the acid of injury which was found to be about pH 5.5.

When NaCl and KCl (pH 7) are injected into amebæ colored with phenol red, no immediate change in color occurs. If the concentration of these salts is lethal (2) the rounded amebæ gradually take on the color indicative of the pH of the environment. Injection of non-lethal concentrations of these salts results during the quiescent period in a slight shift in color toward that suggestive of the pH of the surrounding medium whether this be acid or alkaline. But as soon as the ameba recovers the color reverts to the normal orange-yellow.

The change in color of the discarded spheres after HCl and $CaCl_2$ had been injected or of the dead ameba when lethal amounts of NaCl or KCl were introduced is due to the penetration from the environment. It is quite obvious that any uncontrolled changes in the environmental pH would be confusing. Therefore it was necessary to take precautions to obviate this factor. In preliminary experiments, when ordinary cover slips were used the medium increased in alkalinity markedly during its contact with the coverslip. The use of pyrex glass or coverslips coated with balsam or collodion did not prevent this change in hydrogen ion concentration. To maintain a constant pH of the environment a buffer calcium acetate solution [1] of pH 6 was used. In this amebæ were immersed and the various salts and acids injected. In this case the dead spheres and the amebæ killed with NaCl or KCl took on the color representing the reaction of the surrounding medium, viz., pH 6. With quartz cover slips, which do not affect the pH of solutions coming into contact with them, the medium remained constant and the discarded spheres and dead amebæ assumed the reaction of any environing medium into which they were placed. These results show that the injection of the individual chlorides are ineffective in changing the intracellular pH except when toxic concentrations were used.

Discussion.

In the marine egg the production of acid due to injury is much more easily manifested than in the ameba. As previously shown (4) a localized flash of color indicating acid production is apparent in the starfish egg if the needle is introduced abruptly

[1] We wish to thank Dr. William Perlzweig for the preparation of this buffer solution.

into the interior. This change is not evident in the ameba unless the mechanical trauma is vigorous enough to cause death of the disturbed part. This difference points to a greater susceptibility to injury of the egg or an increased buffering power of the ameba which may in turn be due either to an increase in the amount of buffer present or to a greater ease in diffusion of buffers through the cell. This faster rate of mobilization of buffers in the ameba as compared to the egg is suggested by the constant flow of cytoplasm of the ameba in contrast to the relatively quiescent cytoplasm of the egg.

The production of acid when $CaCl_2$ or $MgCl_2$ is introduced into the ameba may be due to the production of insoluble Ca or Mg salts with the liberation of free acid. Aub and Reznikoff (7) have suggested such an explanation for the effect of Pb salts on red blood cells. Ca may also unite with the carbonate and phosphate to form insoluble salts with the production of free acid. This acid formation is evident until some alkali diffuses into the solidified mass and neutralizes the acids present. Such an explanation does not preclude the possibility also of the formation of a Ca or Mg organic compound.

Conclusions.

1. The cytoplasm of the living *Amœba dubia* shows considerable buffering power to pH changes induced by the injection of salts and buffers.

2. If HCl, injected into the ameba, is immediately buffered by the cytoplasm no toxic effect results. If the quantity injected is too great to be buffered, the affected portion of the cell dies and is discarded.

3. $CaCl_2$, $MgCl_2$ and $AlCl_3$, injected into amebæ colored with indicators, give colorimetric evidence of the production of acid greater in amount than can be explained by acid produced by mechanical injury. Unless this color reverts immediately to that indicative of normal cytoplasm, the affected portion is discarded in the case of $CaCl_2$ and $AlCl_3$ and the entire cell dies in the case of $MgCl_2$.

4. Upon death permeability changes occur so that the dead mass of the ameba quickly assumes the hydrogen ion concentration of the environment.

BIBLIOGRAPHY.

1. **Chambers, R., Pollack, H., and Hiller, S.**
'27 The Protoplasmic pH of Living Cells. Proc. Soc. Exp. Biol. and Med., xxiv, 760.

2. **Chambers, R., and Reznikoff, P.**
'26 Micrurgical Studies in Cell Physiology. I. The Action of the Chlorides of Na, K, Ca, and Mg on the Protoplasm of *Amœba proteus*. J. Gen, Physiol., viii, 369.

3. **Clark, W. M.**
'25 The Determination of Hydrogen Ions. Williams and Wilkins Co., Baltimore, 2d ed., 81.

4. **Chambers, R., and Pollack, H.**
'27 Micrurgical Studies in Cell Physiology. IV. Colorimetric Determination of the Nuclear and Cytoplasmic pH in the Starfish Egg. J. Gen. Physiol., x, 739.

5. **Reznikoff, P.**
'26 Micrurgical Studies in Cell Physiology. II. The Action of the Chlorides of Lead, Mercury, Copper, Iron, and Aluminum on the Protoplasm of *Amœba proteus*. J. Gen. Physiol., x, 9.

6. **Chambers, R.**
'29 Intracellular Hydrion Concentration Studies, I. The Environment and the pH of Cytoplasm and of Inclusion Bodies. Biol. Bull., LVI, 369.

7. **Aub, J. C., and Reznikoff, P.**
'24 Lead Studies III. The Effects of Lead on Red Blood Cells. Part 3. A Chemical Explanation of the Reaction of Lead with Red Blood Cells. J. Exper. Med., xl, 189.

INTRACELLULAR HYDRION CONCENTRATION STUDIES.

III. The Buffer Action of the Cytoplasm of *Amœba dubia* and Its Use in Measuring the pH.

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Recent determinations of intracellular pH have been made by noting the color of indicator dyes injected directly into the protoplasm (1, 2, 3, 4, 5). The recorded value was found by comparing the results of injecting a series of overlapping dyes. The color of the dye, whose range was found to include the pH of the cytoplasm, was compared with known standards projected optically into the field of the microscope.

While attempting to determine the buffer action of the cytoplasm it was found that an indirect method could be used to check the results obtained from the direct color comparisons.

It is known that a drop of a solution at a certain pH added to another buffer solution containing an indicator dye will cause a momentary localized change in the color providing the reactions of the two solutions are different. The closer the pH values of the two solutions are to one another, the less marked will be the color change. When they have the same pH there will be no change in color. It is possible to take advantage of this fact in measuring the intraprotoplasmic pH by injecting a series of solutions of known pH into cells colored by the previous injection of indicator dyes. As will be brought out later, this technique is only approximate but serves to check wide deviations from direct tint comparisons.

It has been shown that $M/4$ solutions of mono-sodium phosphate may be injected with no toxic effect (6), and that the potassium ion has about the same toxicity as the sodium ion on injection (7). Hence the Clark buffer solutions (8) whose KH_2PO_4 concentration is $M/20$ should be non-toxic from the point of view of salt concentrations, and any toxic effect must be due to the buffered hydrogen ion concentration.

Amœba proteus and Amœba dubia were used in this study since their pH has been determined by direct tint readings.

The amebæ were injected with brom cresol purple and phenol red which were the indicators whose ranges cover the pH as found by previous work. The colored amebæ were then injected with the phosphate buffers from pH 5.6–8.0 and observations made on the changes in color.

When buffer solutions of 5.6, 5.8, 6.0 were injected into amebæ colored with brom cresol purple, a temporary but distinct yellow flash was produced. Those above 6.2 produced no color change with this indicator which is already blue in the cell. Buffer solutions of pH 6.2 and 6.4, when injected into amebæ colored orange yellow with phenol red, gave temporary yellow flashes. With the same indicator, solutions having a pH of 6.6, 6.8 or 7.0 showed no color change. Those whose pH was 7.2 and above showed reddish flashes in the orange yellow colored cytoplasm.

This shows that the pH of the amœba is not less than 6.6 and not greater than 7.2. This is in accord with the results obtained in this laboratory in previous investigations and not with those obtained by the Needhams (2). They also used the microinjection technique with direct color comparison for reading the pH values. Their value for the cytoplasmic pH of the amœbæ was 7.6, as was Pantin's, who used the neutral red vital staining technique (9).

As for the Needhams' results it must be remembered that they were using a European species and also that they report the amebæ died within five minutes after injection. In the investigation reported in this paper the amebæ were allowed to recover fully after the injection before treatment with the buffer solutions. With a proper injection of phenol red and brom cresol purple, amebæ can be kept alive and apparently normal for at least two days (4). Frequent checks on the color by direct comparison with standard buffers showed no change during that time. The amebæ colored with phenol red maintained the same orange yellow tint (pH 6.9 ± 0.1) as long as they were kept under observation. On the other hand moribund and dead amebæ take on the pH of the environment which is usually alkaline when the ordinary glass coverslips are employed without proper

precautions (5). As for the value obtained by Pantin (8) the inefficacy of neutral red staining has been shown (3, 10).

The interesting fact is that regardless of the pH value of the buffer solution injected the return of color of the indicator present to its usual one is quite rapid and constant. If, however, sufficient buffer was put in to change the pH of the cell, the cell died. These facts emphasize two important points relating to intracellular hydrogen ion concentration. One, that the cytoplasm has a considerable buffering power, and two, that when the pH of the cytoplasm is changed, the cell dies.

BIBLIOGRAPHY.

1. **Needham, J., and Needham, D. M.**
   '25–'26 The Hydrogen Ion Concentration and Oxidation-reduction Potential of the Cell Interior before and after Fertilization and Cleavage: A Micro-injection Study of Marine Eggs. Proc. Roy. Soc. London, Series B, XCIX, 173.
2. **Needham, J., and Needham, D. M.**
   '26 Further Micro-injection Studies on the Oxidation-reduction Potential of the Cell Interior. Proc. Roy. Soc. London, Series B, XCIX, 383.
3. **Chambers, R., and Pollack, H.**
   '27 Micrurgical Studies in Cell Physiology, IV. Colorimetric Determination of the Nuclear and Cytoplasmic pH in the Starfish Egg. J. Gen. Phys., X, 739.
4. **Chambers, R., Pollack, H., and Hiller, S.**
   '27 Protoplasmic pH of Living Cells. Proc. Soc. Exp. Biol. Med., XXIV, 760.
5. **Reznikoff, Paul, and Pollack, H.**
   '29 Intracellular Hydrion Concentration Studies. II. The Effect of Injection of Acids and Salts on the Cytoplasmic pH of *Amœba dubia*. Biol. Bull., LVI, 377.
6. **Reznikoff, Paul, and Chambers, R.**
   '27 Micrurgical Studies in Cell Physiology. III. The Action of CO_2, and Some Salts of Na, Ca, and K on the Protoplasm of *Amœba dubia*. J. Gen. Physiol., X, 731.
7. **Chambers, R., and Reznikoff, P.**
   '26 Micrurgical Studies in Cell Physiology. I. Action of the Chlorides of Na, K, Ca, and Mg on the Protoplasm of *Amœba proteus*. J. Gen. Phys., VIII, 369.
8. **Clark, Wm. M.**
   '22 The Determination of Hydrogen Ions. Williams and Wilkins Co., Baltimore, Md.
9. **Pantin, C. F. A.**
   '23 On the Physiology of Ameboid Movement. J. Marine Biol. Assn., XIII, 1, 24.
10. **Chambers, Robert.**
    '29 Intracellular Hydrion Concentration Studies. I. The Relation of the Environment to the pH of Protoplasm and its Inclusion Bodies. Biol. Bull., LVI, 369.

THE EFFECTS OF CHANGES IN MEDIUM DURING DIFFERENT PERIODS IN THE LIFE HISTORY OF *UROLEPTUS MOBILIS* AND OTHER PROTOZOA.

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3. The Effects of Yeast Extracts.

The effect of vitamines on the vitality of protozoa has been a subject of but little investigation. In 1917 Calkins and Eddy (1) reported no effect of treating paramecia with pancreatic vitamine extracted with Fuller's earth. In 1918 Lund (2), working with yeast extracts, found that if Paramecia had been starved before being fed with boiled yeast their size and speed of oxidation were increased but not the cell division. In 1919 Chambers (3) reported a slight increase when the animals were fed yeast, especially ground yeast, and in the same year Flather (4) obtained similar results with the unpolished rice. All of these experiments were upon *Paramecium*, which is not a favorable subject for investigation unless pure lines are established and endomixis watched, for a change in the vitality may be due to a reorganization of the protoplasm rather than to a change in the environment. Abderhalden and Kohler in 1919 (5) reported a slight stimulation of *Colpoda cucullus* when treated with yeast extracts but the evidence is not decisive.

Through the courtesy of Professor W. H. Eddy and Dr. Ralph Kerr, of Teachers College, I have had placed at my disposal the following yeast extracts. (1) Alpha bios No. 223 extracted by Professor Eddy in 1924 (6). (2) Beta bios isolated in 1928 by Dr. Kerr (7). (3) Gamma bios a residue substance similar to bios II reported by Lucas and Miller (8) in 1924. These three substances were prepared as indicated in Table I., which has been compiled by Dr. Kerr.

Table I.

The Separation of Yeast Autolyzate into Various Bios Factors.

I. *Preliminary Fractionation.*

1. Make autolyzed yeast 66 per cent. alcoholic by volume to precipitate proteins. Filter.
2. Filtrate from 1. Add hot saturated baryta so long as an immediate precipitate

forms. Add alcohol as necessary to maintain 66 per cent. strength. Filter. Save ppt. for 8.

3. Filtrate from 2. Contains alpha bios and some gamma bios. Neutralize immediately with sulfuric. Adjust to pH 4.7. Precipitate with iron sol. and discard pptate.

4 Filtrate from 3. Adjust to pH 5.3 Precipitate with iron sol. Filter. Save filtrate for 7. Precipitate contains all the alpha bios.

II. *Isolation of Alpha Bios.*

5 Ppt. from 4. Work up with water and refilter to remove water washings. Dissolve ppt. in 30 per cent. sulfuric. When solution is complete dilute with water and neutralize with baryta. (Fe, SO_4, ions removed as $Fe(OH)_3$ and $BaSO_4$). Filter by suction and discard ppt. With baryta and sulfuric remove quantitatively all Ba, Fe and SO_4 ions.

6. Filtrate from 5. Evaporate to dryness and recrystallize from hot 95 per cent. ethyl alcohol. Purified product has melting point 223° C.

III. *Concentration of Gamma Bios.*

7. Start with filtrate from 4 Evaporate to small volume. Add sulfuric to make 5 per cent. by weight. Filter if necessary and discard ppt. Now add phosphotungstic dissolved in 5 per cent. sulfuric so long as any ppt. forms. Filter and discard filtrates. Ppt. contains gamma bios.

15. Start with phosphotungstates from 7 and 12. Decompose with baryta in the usual way. Filter. Make filtrate decidedly alkaline to litmus. Add alcohol to 80 per cent. by volume. Filter and discard ppt. if any. Free filtrate of Ba and SO_4 quantitatively. Filtrate now contains a product not yet purified but which suggests Miller and Lucas' bios II. We designate it here as gamma bios.

IV. *Isolation of Beta Bios.*

8. Start with ppt. from 2. Wash with alcohol. Then stir washed ppt. repeatedly with water and filtrate by suction so long as the water is colored. Neutralize the filtrates immediately with sulfuric. Refilter and discard pptates and residue.

9. Filtrate from 8. Treat with hot saturated Ag_2SO_4. Filter and discard ppt.

10. Filtrate from 9. Treat with hot saturated acid mercuric sulfate. Filter. Discard ppt.

11. Filtrate from 10. Free from Ag and Hg ions with H_2S. Remove excess SO_4 with baryta. Save clear filtrates. Evaporate at 40° C. to small volume.

12. Filtrate from 11. Make 5 per cent. sulfuric by weight and extract five times with ether of equal volume discarding ether extract. Add phosphotungstic acid in 5 per cent. sulfuric until no further ppt. forms. Filter. Add the ppt. to 7. (See III, 7 above.) Make filtrate slightly alkaline with baryta and refilter. Discard this ppt.

13. Filtrate from 12. Neutralize with sulfuric and evaporate to small volume. Make 80 per cent. alcoholic by volume and again ppt. with baryta. Filter and discard filtrate.

14. Precipitate from 13. Free of Ba with sulfuric. Evaporate to a thick sirup at 40° C. Dehydrate by stirring and grinding with dry acetone to a fine white powder. Filter nearly to dryness on suction filter but leave enough acetone to make a moist mass. Transfer acetone-wet product to vacuum desiccator and here free of acetone by suction. Product is *Beta bios.*

I wish to express my appreciation to Professor Eddy and Dr. Kerr for their interest and helpful suggestions.

The work with the bioses was begun in 1926–27 at the same time when experiments with di-sodium phosphate were being conducted on *Uroleptus mobilis* in order to determine any variations in response according to the age of the protoplasm. Since then *Dallasia* from pure lines of Professor Calkins, *Stylonychia*, and *Pleurotricha* have been used in addition to *Uroleptus mobilis*. Whenever possible the material was taken from pure lines started from an exconjugant or cyst so that the age of the protoplasm was known. In the case of *Stylonychia* conjugation did not occur and the material was obtained from a single individual isolated from the wild culture. The methods used in all experiments were the same as those of earlier papers and as usual the rate of division is considered an indication of the vitality of the protoplasm.

In earlier papers (9, 10), results of experiments have been reported which indicate that the protoplasm of *Uroleptus mobilis* varies in its response to treatment. Beef extracts and di-potassium phosphate cause an increase in the division rate only when the protoplasm is mature while di-sodium phosphate causes an increase in the division rate of cells of all ages but the greatest increase occurs in the mature cells. Experiments with di-sodium phosphate have been continued and will serve as an additional control in the majority of the experiments with the bioses.

I. *Experiments with Alpha Bios.*

Three series of *Uroleptus mobilis* were used in these experiments. Various concentrations of alpha bios were tried and finally a concentration of .05 mg. per cc. was fixed as the best. The bios solution was added to the normal hay flour medium daily and controls were carried on in the normal medium and also parallel experiments were conducted at the same time with a medium containing di-sodium phosphate in the Packard (11) concentration of $M/7000$. The results are shown in Table II.

TABLE II.

EFFECTS OF DI-SODIUM PHOSPHATE AND ALPHA BIOS ON THE DIVISION RATE OF *Uroleptus mobilis*.

Series No.	Age in Gen.	Amount of Variation from Control in Division Rate per Line in 10-day Periods.	
		Di-sodium Phosphate Series.	Alpha Bios Series.
139	19	+ 3.8	—
	64	+ 3.4	—
	125	+ .6	− 3.0
	175	+ .6	− 2.4
	225	+ 4.4	+ 3.0
	240	+ 1.6	− 2.6
	275	+ 3.4	+ 3.0
140	19	+ .4	—
	60	+ 2.4	+ 1.8
	117	− 3.0	− 3.2
	157	− 1.0	− 4.8
	218	+ 3.4	+ 2.0
	227	+ 4.2	+ .6
141	6	+ 1.2	− 1.8
	36	− 1.0	− 2.2
	66	+ 1.8	− 2.2
	125	+ 1.6	− 2.4
	180	+ 4.2	+ 1.9
	192	+ 4.4	+ 2.4
	234	+ 3.2	+ 4.0
	243	− .6	− 4.0

As in former experiments the sodium phosphate caused a stimulation of the vitality throughout the life history of Series 139 and practically throughout the life of Series 141. Series 140 was the least vigorous and died out in the 227th generation after showing instability throughout its life. The greatest stimulation however in all three series appeared during maturity.

Alpha bios failed to act as a stimulant save in the 225th and 275th generation of Series 139, three times at slightly irregular intervals in Series 140 and only slightly after the 180th generation in Series 141 save in the 234th generation when there was a slightly higher division rate than that of the control or sodium series. Thus alpha bios with few exceptions has a depressing effect on the vitality of these three series of *Uroleptus*.

These results may be due to at least two factors: (1) too acid a

condition of the medium, (2) a general lowering of the vitality of *Uroleptus mobilis*. Undoubtedly the protoplasm was weakening as it did not respond as vigorously to sodium stimulation as it did in the experiments of 1926. On the other hand the H ion concentration of the alpha bios medium was slightly lower than that of the normal medium (7.2). Since trial experiments with beta bios known to be more acid, resulted in a decided lowering of the vitality and since it seemed uncertain whether any bios would cause a definite stimulation of an animal cell, experiments were conducted in which a yeast extract containing all the bioses was used and in one series di-sodium phosphate was added to the yeast extract medium to increase the alkalinity.

II. *Experiments with Harris Yeast Extract.*

Four experiments with *Uroleptus* at varying ages and two with *Dallasia* were carried on in which the animals were kept (1) in a normal medium to which was added daily yeast extract of a concentration of .01 mg. per cc., (2) in a normal medium to which was added di-sodium phosphate, (3) in the same medium as in (2) with the addition of the yeast extract and (4) in normal hay flour medium as the control series. These results are shown in Table III.

TABLE III.

EFFECTS OF YEAST EXTRACT ON THE DIVISION RATE OF *Uroleptus mobilis* AND *Dallasia*.

Series No.	Age in Gen.	Amount of Variation from Control in Division Rate per Line in 10-day Periods.		
		Sodium Phosphate Series.	Yeast Extract Series.	Sodium Phosphate Yeast Extract Series.
Uroleptus				
146....	15	died	− .2	died
143....	30	− 2.0	− 3.6	+ .4
142....	84	− 3.4	− 2.8	− 2.6
141....	250	0.0	− 5.2	− 2.8
Dallasia				
1.....	75	− 3.4	+ 5.0	+ 9.0
2.....	75	+ 3.2	+ 7.2	+ 10.4

The experiments with *Uroleptus* show practically no stimulating effect of the yeast extracts. The protoplasm was too weak to respond and the entire race died out shortly afterwards. The two experiments with two different series of *Dallasia* both in the 75th generation, showed a definite increase in division rate in all the experimental series. It was especially marked in the yeast sodium hay-flour medium when the rate was 10.4 divisions higher than that of the normal control series and 12.4 higher than the sodium hay-flour series for the same period. Since the yeast extracts caused a marked stimulation of the vitality of *Dallasia* both with and without the addition of sodium to the normal medium the fractional extracts of the yeast were then used.

III. *Experiments with a Neutral Salt of Beta Bios and with Gamma Bios.*

Dallasia, *Pleurotricha* sp. and *Stylonychia* sp. were treated with the two bioses using the same methods as above. These three animals differ in their normal rate of cell division. *Dallasia* when young undergoes from 3–5 divisions daily, *Pleurotricha* like *Uroleptus* not more than 1–2 divisions and *Stylonychia* divided every other day. *Stylonychia* may have been more mature as in this series no conjugations occurred and the age is unknown. The results of the experiments are shown in Table IV.

TABLE IV.

THE EFFECTS OF BETA AND GAMMA BIOS ON THE DIVISION RATE.

Series No.	Age in Gen.	Amount of Variation from the Control in Division Rate per Line in 10-day Periods.	
		Beta Bios Series.	Gamma Bios Series.
Dallasia 2.....	115	− .8	+ 4.8
	130	+ .2	+ 4.8
Pleurotricha	90	+ 5.0 (1st 10 days)	+ 10.2 (1st 10 days)
		died (2d " ")	+ 1.6 (2d " ")
	110	—	+ 4.8
Stylonychia		—	+ 3.8 (1st 10 days)
		—	+ 4.8 (2d " ")

Beta bios apparently had no effect on *Dallasia* when older gamma bios, however acted as a definite stimulant increasing the division rate, 4.8 divisions per line in 10 days. *Pleurotricha* was stimulated in the 90th and 110th generation and showed a marked response to gamma bios. *Stylonychia* had its division rate almost doubled in the gamma bios solution and this effect continued for twenty days. When *Pleurotricha* was stimulated for twenty days the effect died out during the second ten-day period. This may be correlated with the variation in normal vitality of the two species.

IV. *Experiments with Alpha, Beta and Gamma Bios.*

Finally experiments were conducted to compare the effects of the three bioses on *Pleurotricha* and *Stylonychia*, when added to the normal medium and in a few experiments to the sodium medium. The results are shown in Table V.

TABLE V.

EFFECTS OF ALPHA, BETA, AND GAMMA BIOS ON THE DIVISION RATE OF *Stylonychia* AND *Pleurotricha*.

	Amount of Variation from the Control in the Division Rate per Line in 10-day Periods.					
Alpha Bios Series.	Beta Bios Series.	Gamma Bios Series.	Na_2HPO_4 Series.	Na_2HPO_4 Alpha Bios.	Na_2HPO_4 Beta Bios.	Na_2HPO_4 Gamma Bios.
Stylonychia						
+ 1.4.....	+ 4.2	0.0	. + 4.4	—	—	+ 3.2
Pleurotricha						
115 gen +10.2	+ 11.4	+ 10.2	+ 8.2	—	—	+ 16.0
130 gen. died..	+ 4.8	+ 8.0	− .8	died	9.0	+ 12.8

In these experiments, *Stylonychia* quickened its division rate in all media save that of the Gamma Bios, where the division rate equalled that of the control. *Pleurotricha* in the 115th generation showed a definite stimulation in all media, especially in that with the HNa_2PO_4 and gamma bios. In the second experiment the division rate of the control dropped to 5.4 divisions per line in 10 days while the experimental series kept a much higher level, the climax being reached with 18.2 divisions per line for the same 10-day period in the series kept in normal

medium to which HNa_2PO_4 and Gamma Bios solution had been added.

Summary and Conclusions.

These preliminary experiments indicate that while the protoplasm of *Uroleptus mobilis* was usually depressed when treated with yeast extracts due probably to its weakened condition, that of *Dallasia*, *Pleurotricha* and *Stylonychia* were definitely stimulated by the addition of fractional extracts of yeast to the normal medium. Alpha bios in general causes the least effect and gamma bios the greatest increase in division rate. While there is usually an increase in the protoplasmic activity when sodium phosphate is added to the medium already containing the bios solution, this may not be due to an increased alkalinity as the variation in Hydrogen ion concentration were not more than .1–.2 of a point. The explanation may lie in an increase in the permeability of the cell allowing a far more reaching effect of the bios solution. The age and general characteristics of the protoplasm must also be taken into consideration and further experiments are planned with pure lines of varying ages.

The fact of a sudden marked increase in the division rate of a protozoan cell when treated with these yeast extracts brings to mind the theory of Burrows in which the rapid growth of cells and formation of tumors is assumed to be due to a lack of balance between vitamines in the cells. The relation of these extracts to vitamines has yet to be proved. They are however stimulating substances to yeast cells and to certain animal cells, their effects varying according to the age and general conditions of the protoplasm.

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References.

1. **Calkins, G. N., and Eddy, W. H.**
'17. Soc. Exp. Biol. and Med., Vol. XIV., 162.
2. **Lund.**
'18 Am. Jour. Phys., Vol. XLVII., p. 167.
3. **Chambers, B.**
'19 Biol. Bull., Vol. XXXVI., p. 82.
4. **Flather.**
'19 Biol. Bull., Vol. XXXVI., p. 54.
5. **Abderhalden and Kohler.**
'19 Arch. f. ges. Physiologie, Vol. CLXXVI., p. 209.

6. **Eddy, W. H., Kerr, and Williams.**
'24 Jour. Am. Chem. Soc., Vol. XLVI., 2846.
7. **Kerr, R. W.**
'28 Soc. Exp. Biol. and Med., Vol. XXV., 3847.
8. **Lucas and Miller.**
'24 Jour. Phys. Chem., Vol. XXVIII., 1180.
9. **Gregory, L. H.**
'25 BIOL. BULL., Vol. XLVIII., No. 3.
10. **Gregory, L. H.**
'26 BIOL. BULL., Vol. LI., No. 3.
11. **Packard, C.**
'26 Jour. Cancer Research, May.
12. **Burrows.**
'26 Soc. Exp. Biol. and Med., Vol. XXIV., 3240.